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Physiological responses of the dragonfly Libellula julia uhler exposed to aluminum in soft water at low pH.

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PHYSIOLOGICAL RESPONSES OF THE DRAGONFLY *LIBELLULA JULIA* UHLER EXPOSED TO ALUMINUM IN SOFT WATER AT LOW PH

A Dissertation Presented

by

JOHN PHILIP ROCKWOOD

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To my

Parents

and Brother

ACKNOWLEDGEMENTS

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ABSTRACT

PHYSIOLOGICAL RESPONSES OF THE
DRAGONFLY LIBELLULA JULIA UHLER EXPOSED
TO ALUMINUM IN SOFT WATER AT LOW PH

MAY 1989

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The tolerance and physiological responses of last instar nymphs of the dragonfly Libellula julia (Odonata: Anisoptera) exposed to low pH and elevated aluminum concentrations at low pH in soft water were investigated. The tolerance of L. julia to these toxicants was evaluated using acute (96 hour) and subchronic (90 day) toxicity tests. The nymphs were found to be extremely tolerant of both low pH and aluminum at acidic pHs.

The physiological responses -- acid-base status, respiration, water balance, and ionic regulation -- were evaluated following a 96 hour exposure to combinations of pH (2.3 and 4.0) and aluminum (0, 0.3, 3, and 30 mg/l). The parameters examined were wet, dry, and ash weights; body burdens of Na^+, K^+, Ca^{2+}, and Mg^{2+}; hemolymph osmolality and concentrations of the above cations and Cl^-; hemolymph volume; hemolymph pH; and oxygen uptake.

The observed responses were dependent upon both the pH and the aluminum concentration of the test solution. Exposure to pH 4.0 caused no significant physiological disturbances. The addition of aluminum at this pH resulted in depressed hemolymph pH levels (30 mg Al/l) and greatly reduced oxygen uptake rates (3 and 30 mg Al/l) relative to the control. Exposure to pH 2.3 provoked significant reductions in wet and ash weights, hemolymph osmolality, and hemolymph pH. The addition of 30 mg Al/l greatly exacerbated the above effects. Furthermore, the body burden of Na^+, hemolymph concentrations of Na^+ and Cl^-, and hemolymph volume were found to be drastically reduced relative to both the control and the pH 2.3 treatment. Additionally, the Ca^{2+} body burden was significantly depressed, but only compared to the pH 2.3 treatment. Following a 192 hour exposure to pH 2.3, reduced hemolymph Na^+ and elevated hemolymph K^+ levels were observed. More moderate aluminum levels (0.3 and 3 mg/l) at this pH elicited responses very similar to those found in the pH 2.3 treatment.

Of the responses examined, oxygen uptake proved to be the most sensitive indicator of stress, while ionic regulation was most closely correlated to mortality. The extent and significance of these alterations are discussed.

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CHAPTER I

INTRODUCTION

Acid precipitation is a widespread, ecological problem adversely affecting both terrestrial and aquatic ecosystems. Presently, it is seriously impacting the northeastern United States (Likens and Bormann, 1974; Schofield, 1976; Last et al., 1980), the Canadian Shield (Beamish and Harvey, 1972; Beamish, 1974), the United Kingdom (Gorham, 1976), and southern Scandinavia (Jensen and Snekvik, 1972; Wright and Gjessing, 1976; Wright et al., 1976). The principal cause of acid deposition is the combustion of fossil fuels which releases sulfur dioxide and nitrogen oxides into the atmosphere (Likens et al., 1979; Cowling, 1982). The long distance transport of these oxides (and their transformation products sulfuric and nitric acids), due to the increased use of taller smoke stacks, has transformed local problems into regional and possibly global problems (Likens et al., 1979; Cowling and Linthurst, 1981; Haines, 1981; Chadwick, 1983).

These acids alter water chemistry both directly, through deposition on lake and stream surfaces, and indirectly through the mobilization of metals and other materials from the terrestrial to the aquatic environment (Wright and Gjessing, 1976; Dickson, 1980). Because of their larger relative surface area, terrestrial systems intercept the bulk of atmospheric deposition before it reaches aquatic ecosystems (Dillon et al., 1984). Therefore, the soils and bedrock of the watershed play an important role in determining the

sensitivity of a water body to acid precipitation (Johnson et al., 1981) as well as its chemical composition (Wright and Gjessing, 1976).

The coarse, noncalcareous, glacial till and slowly weathering bedrock, common in eastern North America and northern Europe, provide little buffering capacity, hence limited protection for lakes and streams (Gorham, 1976; Wright et al., 1976; Glass et al., 1980; Hendrey et al., 1980). Consequently, when acid rain is deposited on soils poor in basic cations (e.g., Ca^{2+} and Mg^{2+}), hydrolysis of silicon-oxygen-aluminum linkages results, releasing large quantities of aluminum (dissolved) into the soil solution (Voigt, 1980; Hellawell, 1986). This, the first of a two-step neutralization process, occurs primarily in the soil zone, and serves to rapidly neutralize hydrogen ion acidity through the dissolution of preexisting aluminum hydroxide compounds and the leaching of bases from organic matter and various biological systems (Johnson, 1979). In a much slower second step, the chemical weathering of silicate materials in the soil and bedrock releases alkali and alkaline earth cations which neutralize both hydrogen ion and aluminum acidity (Johnson et al., 1981). During the neutralization process, however, a large portion of this soil-derived aluminum is leached/mobilized from the watershed to acidified water bodies (Cronan and Schofield, 1979) with dire consequences for the indigenous aquatic biota (Driscoll et al., 1980; Campbell and Stokes, 1985).

In north-temperate, aquatic ecosystems, a factor of considerable importance is the sudden, episodic additions of impurities associated with the spring snowmelt (Gorham, 1976; Dillon et al., 1984). Laboratory and field studies indicate that in excess of 50% of the sulfate, nitrate, and hydrogen ions originally present in the snowpack is released in the first 10% to 35% of the melt water (Johannes et al., 1980; Johannessen et al., 1980; Semkin and Jeffries, 1986). Because the shock effect precludes any opportunity for acclimation, aquatic organisms are affected to a greater extent by short term, episodic events (i.e., sudden pulses of both hydrogen ions and aluminum), than by long term, gradual, acidification processes (Dillon et al., 1984; Harvey and Whelpdale, 1986). Results of snowmelt simulation experiments in streams have shown that episodic increases in hydrogen ion and/or aluminum concentrations adversely affect the structure and function of fish and benthic macroinvertebrate communities (Hall et al., 1985, 1987; Ormerod et al., 1987; Weatherley et al., 1988; McCahon and Pascoe, 1989). Furthermore, laboratory investigations have found repetitive, intermittent exposures to aluminum under acidic conditions to be more toxic to brook trout than continuous exposures (Siddens et al., 1986).

The acidification of surface waters has been found to affect all of the major trophic levels of aquatic organisms (Hendrey et al., 1976; Wright et al., 1976; Haines, 1981; Okland and Okland, 1986; Schindler, 1988). The most obvious and economically devastating, however, is the damage to and widespread extinction of

commercial and recreational fisheries (Beamish, 1974). The fish population structure in acid impacted waters is dominated by older, larger adults (Schofield, 1976; Harvey, 1980). This is due to recruitment failure caused by increased egg and larval mortality (Jensen and Snekvik, 1972; Johansson et al., 1973) and/or inhibition of spawning by adults (Beamish et al., 1975). Consequently, fish have been the subjects of a majority of the physiological studies on the impact of acidification on aquatic organisms.

Although fish have been the focus of much research, studies indicate that acidification causes ecologically important reductions in the diversity of primary producers, decomposers, zooplankton, and macroinvertebrates (Hendrey et al., 1976; Wiederholm and Eriksson, 1977; Fiance, 1978; Stokes, 1986). Similarly, experimental acidification of lotic and lentic systems resulted in increased invertebrate drift (lotic), decreased diversity, increased abundance of tolerant forms, and decreased complexity of the food web (Hall et al., 1980; Schindler et al., 1985). These changes in community structure can be brought about both directly through pH and/or aluminum toxicity, and indirectly via the alteration of predation schemes caused by the elimination of fish, an acid sensitive top predator (Eriksson et al., 1980; Henrikson et al., 1980; Bendell, 1986). At this time, however, the physiological responses of aquatic macroinvertebrates, and more specifically aquatic insects, to elevated hydrogen ion and aluminum concentrations remain largely undescribed.

Objective

The objective of this study is to describe selected physiological responses of last instar nymphs of the dragonfly Libellula julia exposed to low pH and aluminum at low pH. Acute and subchronic toxicity as well as water balance, ionic regulation, acid-base status, and oxygen consumption will be examined as indices of pH/aluminum stress. Accordingly, the following experimental procedures will be implemented:

1) Separate acute 96 hour LC50s will be determined (if possible) for low pH and aluminum. These values will permit identification of sublethal/threshold levels to be used in the physiological studies.

2) Median survival times and percent survival will be determined following a 90 day subchronic exposure to a variety of pH/aluminum combinations. The intent here is to relate the observed physiological changes with mortality.

3) Gross changes in body composition will be studied by measuring wet, dry, and ash weights. In those treatments where a substantial change in wet weight is observed, hemolymph volume and the change in wet weight through time will be examined.

4) Ionic regulation will be studied by measuring the body burdens of Na^+, K^+, Ca^{2+}, and Mg^{2+}. In addition, hemolymph osmolality and concentrations of the above cations and Cl^- in the hemolymph will be determined.

5) Acid-base status will be examined by measuring hemolymph pH levels following exposure to a wide range of pH/aluminum combinations.

6) Oxygen consumption of individual nymphs will be determined at ecologically significant pH and aluminum levels.

CHAPTER II

LITERATURE REVIEW

In acidified waters, several interrelated physiological functions of aquatic organisms are impaired: Na^+ and Ca^{2+} regulation, acid-base status, and respiration (Fromm, 1980; Haines, 1981; Havas, 1981; McDonald, 1983; Okland and Okland, 1986). The mechanisms affected, the relative degree of disruption, and consequential mortality depend upon a complex interaction between environmental pH and ambient concentrations of aluminum and calcium (McDonald et al., 1980; Muniz and Leivestad, 1980a; Brown, 1981; Howells et al., 1983; Wood et al., 1988).

At moderately low pH levels (pH 4 to 5), it is generally accepted that the primary cause of fish mortality is the failure of body salt regulation (Packer and Dunson, 1972; Leivestad and Muniz, 1976; Schofield, 1976; Ultsch et al., 1981). Net body losses of Na^+ (Cl^-) are largely brought about through abrupt increases in passive ionic efflux and, to a lesser extent, a fairly complete inhibition of active influx components (Packer and Dunson, 1970; McWilliams and Potts, 1978; McWilliams et al., 1980; Hobe et al., 1984b; Freda and McDonald, 1988). Reduced serum Ca^{2+} concentrations in female fish (Beamish et al., 1975; Wiener et al., 1985) as well as transitory changes in Ca^{2+} fluxes (Hobe et al., 1984a) have also been observed. In this regard, low ambient pH levels have been found to alter the activities of a number of gill ATPases (Na^+-K^+-, Na^+-K^+-Mg^{2+}-, Ca^{2+}-, and HCO_3^--)

(Nieminen et al., 1982; McKeown et al., 1985; Powell and McKeown, 1986).

As a consequence of electrolyte loss across the gills, a redistribution of body water, from the extracellular to the intracellular fluid compartments, has been observed in fish (McDonald and Wood, 1981; Milligan and Wood, 1982; Lee et al., 1983; Hobe, 1987). The reduction in plasma volume is believed to be a secondary homeostatic tactic to osmoregulate by counteracting salt loss in fish exposed to acidic water (Fugelli and Vislie, 1982; Muniz et al., 1987).

In laboratory studies with invertebrates, a strong correlation was found to exist between sensitivity to low pH and difficulties with ionic regulation (Havas, 1981; Hollett et al., 1986). As with fish, acid sensitive crustacean and insect species were unable to maintain high internal levels of Na^+ and Cl^-, whereas tolerant forms showed no significant decline in salt content (Potts and Fryer, 1979; Vangenechten et al., 1980; Havas and Hutchinson, 1983; Havas et al., 1984; Lechleitner et al., 1985; Hollett et al., 1986; Wood and Rogano, 1986; Berrill et al., 1987; Rowe et al., 1988a). Furthermore, an inhibition of Na^+ (Cl^-) influx and a stimulation of Na^+ efflux caused by elevated ambient hydrogen ion concentrations have been observed in crayfish (Shaw, 1960; Wood and Rogano, 1986), Cladocera (Potts and Fryer, 1979; Havas et al., 1984), and insect larvae (Stobbart, 1967; Wright, 1975; Vangenechten and Vanderborght, 1980). Ca^{2+} regulation and uptake (reduced postmolt calcification) have also been found to be

affected in a number of invertebrate taxa, including crayfish, daphnids, copepods, freshwater shrimp, and insect larvae (Malley, 1980; Morgan and McMahon, 1982; Schindler and Turner, 1982; Havas and Hutchinson, 1983; Wood and Rogano, 1986).

Studies of the effects of acidification on aquatic invertebrates reveal marked differences in tolerance between taxa and between different life cycle stages (Okland and Okland, 1986; Maltby et al., 1987). In crayfish for example, individuals of the genus Cambarus are more tolerant of low pH, both as juveniles and adults, than those belonging to the genus Orconectes (Berrill et al., 1985; Hollett et al., 1986). Furthermore, earlier life stages (egg attachment, hatchling, and juvenile) were found to be more sensitive than adults (France, 1984; Appelberg, 1987), while early postmolt stages were more sensitive than later postmolt or intermolt stages (Malley, 1980). Similarly, smaller/earlier life stages of aquatic insects tended to be less tolerant than larger/later instar individuals (Fiance, 1978; Correa et al., 1985; Allan and Burton, 1986; Rowe et al., 1988b). Bell (1971) found the period around emergence to be critical in nine species of insects. Among the insects, odonate nymphs appear to be relatively tolerant to low pH under experimental conditions (Stickney, 1922; Bell and Nebeker, 1969; Bell, 1971; Cruz, 1984; Berrill et al., 1987; Mackie, 1989). This tolerance was also observed in the egg stage as hatching rate and development time were found to be unaffected by exposure to low pH (Hudson and Berrill, 1986; Berrill et al., 1987).

The combined impact of low pH and aluminum on aquatic biota, however, has not received as much attention. Although the chemistry of aluminum is fairly well understood (Smith, 1971; Burrows, 1977; Stumm and Morgan, 1981; Plankey et al., 1986; Tam and Williams, 1986; Backes and Tipping, 1987), only recently, due to difficulties in interpreting its toxicity, has the importance of speciation become apparent (Driscoll et al., 1980; Driscoll, 1984). The toxicity of aluminum is largely a function of pH (Baker and Schofield, 1982; Havas and Likens, 1985a) which dictates the species present through complexation, polymerization, and hydrolysis reactions (Campbell and Stokes, 1985). Furthermore, pH exerts an impact by affecting both the solubility and the degree with which aluminum reacts with organic ligands to produce relatively innocuous complexes (Baker and Schofield, 1980; Driscoll et al., 1980; Burton and Allan, 1986). It is generally accepted that the simple ionized hydroxides, $Al(OH)^{2+}$ and $Al(OH)_2^{+}$, are especially toxic, while polymeric forms as well as organically and inorganically bound complexes are less toxic or essentially harmless (Seip et al., 1984; Courtijn et al., 1987).

Muniz and Leivestad (1980a, 1980b) reported similar responses in fish exposed to aluminum in acidic water as those described for low pH alone: a rapid loss of Na^+ and Cl^-. In the presence of aluminum (compared to low pH alone), these net ionic losses resulted from a slight further inhibition of the Na^+ influx, and more importantly, from a large concentration dependent stimulation of the Na^+ (Cl^-) efflux (Dalziel et al., 1987; Wood and

McDonald, 1987; Booth et al., 1988). The former effect may be explained by a marked reduction in gill Na^+-K^+-ATPase activity (Staurnes et al., 1984). As a consequence of disrupted exchange mechanisms, aluminum was found to be acutely toxic at pH levels (pH > 5) that were not otherwise physiologically limiting (Leivestad et al., 1980). Maximum toxicity to fish was observed in oversaturated aluminum solutions between pH 5.2 and 5.5, although sensitivity was shown to vary with species and life stage (Baker and Schofield, 1982; Cleveland et al., 1986; Kane and Rabeni, 1987). In addition, aluminum exerted greater toxicity to brook trout in solutions at pH 4.8-4.9 than in those at pH 4.4 (Siddens et al., 1986; Wood et al., 1988).

The physiological mechanisms disrupted in fish by exposure to aluminum at low pH were similarly affected in invertebrates (Herrmann, 1987a). In acid tolerant taxa, aluminum was found to be the key additional factor resulting in mortality (Havas and Hutchinson, 1982). Extreme toxicity due to aluminum (Havas and Likens, 1985b), as well as its bioaccumulation (Havas, 1985), were observed in Daphnia magna at pH 6.5, where a solid-phase aluminum hydroxide complex predominated. Among acid sensitive crustaceans and insects, elevated aluminum levels at low pH resulted in increased Na^+ (Cl^-) losses relative to individuals in reference solutions at the same pH (Havas and Hutchinson, 1983; Herrmann, 1987b). Furthermore, acid tolerant insects, normally able to maintain their salt balance at low pH, experienced a net loss of Na^+ and Cl^- (Havas and Hutchinson, 1983).

Havas and Likens (1985a) found that aluminum influenced both the influx and outflux of Na^+ in Daphnia magna, although the rate of each was determined by pH. An inhibited Na^+ influx and an elevated hemolymph Na^+ concentration were observed in the waterbug Corixa punctata following exposure to aluminum at low pH (Witters et al., 1984). In two species of crayfish, low pH/aluminum treatments caused a reduction in their hemolymph Na^+ concentrations (Appelberg, 1985). With regard to these changes, aluminum has been found to be associated with the ion transporting epithelia (chloride cells) of both insects and crustaceans (Havas, 1986). Ca^{2+} regulation was also found to be disrupted in a number of invertebrate species (Havas and Hutchinson, 1983; Havas, 1985). Decreased Ca^{2+} uptake by postmolt crayfish due to acid exposure was further inhibited in the presence of aluminum (Malley and Chang, 1985).

In addition to disrupting the ionic balance of aquatic organisms, exposure to water at low pH can lead to the acidification of internal body fluids due, primarily, to the net influx of hydrogen ions across the gills (Havas, 1981; McDonald, 1983). This phenomenon is accompanied by reduced plasma HCO_3^- concentrations, and has been observed in fish (Packer and Dunson, 1970; Neville, 1979a, 1979b; McDonald and Wood, 1981; Ultsch et al., 1981; Hobe et al., 1984b), crayfish (Morgan and McMahon, 1982; Wood and Rogano, 1986), and insect larvae (Jernelov et al., 1981). In brook trout, no additional reduction in plasma pH, beyond that found in the low pH treatments, was provoked by aluminum exposure

(Walker et al., 1988; Wood et al., 1988). In contrast, Correa et al. (1985) observed a significant increase in the hemolymph pH of dragonfly nymphs exposed to low pH, both with and without added aluminum. While elevated hemoglobin concentrations (providing increased hemolymph buffering capacity) were found to be beneficial to midge larvae exposed to low pH (Jernelov et al., 1981), the same cannot be said for daphnids, where hemoglobin provided no obvious physiological advantage (Havas, 1987).

In fish exposed to severe acid stress (pH $\leq$ 3.5), respiratory disturbances resulting in anoxia have been implicated as the primary cause of mortality (Pruthi, 1927; Packer and Dunson, 1972; Schofield, 1976; Haines, 1981). At these low pH levels, increased mucus secretion/coagulation, hyperplasia and epithelial cell damage, and reduced plasma pH levels have been observed (Westfall, 1945; Daye and Garside, 1976; McCormick et al., 1987) resulting in decreased water to blood diffusion of oxygen at the gills and reduced oxygen carrying capacity of the blood (Packer, 1979; Ultsch et al., 1981). Evidence for respiratory distress at ecologically realistic pH levels (pH $\geq$ 4.0) is lacking (Neville, 1979b; Ultsch and Gros, 1979).

The presence of aluminum at these moderate pH levels, however, has been found to impair respiratory gas exchange via mechanisms similar to those observed for acutely toxic pHs. There are many possible consequences of the precipitation/coagulation of aluminum hydroxide, the adsorption/nucleation of aluminum polymers (Freeman and Everhart, 1971; Baker and Schofield, 1982), and/or the binding

of aluminum to organic anions on the gill´s surface (Wood et al., 1988). These include extensive gill tissue damage (Chevalier et al., 1985; Karlsson-Norrgren et al., 1986; Tietge et al., 1988), mucus clogging of the interlamellar spaces of the gills, respiratory stress (reduced blood oxygen content and elevated levels of blood CO_2 and lactate), hyperventilation, and coughing (Muniz and Leivestad, 1980b; Rosseland, 1980; McCahon et al., 1987; Wood and McDonald, 1987; Walker et al., 1988). Supporting this, aluminum has been found to be concentrated in the gills of fish (Grahn, 1980; Neville, 1985; Lee and Harvey, 1986; Youson and Neville, 1987).

Estimates of oxygen consumption by aquatic insects exposed to low pH and aluminum at low pH are rare and inconsistent. Oxygen uptake by dragonfly nymphs was observed to decrease with increasing hydrogen ion and aluminum concentrations (Correa et al., 1985). These researchers further stated that exposure to aluminum at low pH did not provoke a significant reduction in oxygen uptake compared to that observed for low pH alone. Subsequently, Correa et al. (1986) noted a consistent, but nonsignificant reduction in respiration rates in caddisfly larvae during exposure to aluminum at low pH. In contrast, significantly higher respiration rates were found in three species of mayflies subjected to elevated aluminum levels at low pH (Herrmann and Andersson, 1986).

CHAPTER III

MATERIALS AND METHODS

Sampling Site

Insects

Nymphs of the dragonfly *Libellula julia* (Odonata: Anisoptera) (Uhler, 1857) were chosen for study because they are locally abundant and easily collected, identified, and maintained in the laboratory. Last instar nymphs were collected with a D-frame net from a bog on Route 63 in Montague, Massachusetts and returned to the laboratory for positive identification using the key of Walker and Corbet (1975). Species identification was confirmed on adults reared from nymphs in the laboratory.

The specimens (30 per 40 liter aquarium with no substrate) were acclimated to experimental conditions (photoperiod and temperature) for at least one week in filtered (Fisher P8) bog water at 18 $\pm$ 1 C under a 12:12 L:D photoperiod in a walk-in refrigerator (Bush model WM-23). The water was changed twice weekly and feces and damaged nymphs removed daily. The nymphs were maintained on a daily diet of 3rd to 4th instar larvae of *Aedes aegypti* cultured at room temperature in 1.5 liter plastic containers of 1:2 filtered bog water:distilled water on a diet of 1:1 brewers yeast:lactalbumin. Feeding of the dragonflies was discontinued 48 hours prior to and during experimentation.

Water

Preliminary chemistry of the bog water included pH (Beckman pH meter, model 70; range 5.7-6.7), alkalinity (potentiometric titration for low alkalinity samples; mean 2.5 mg $CaCO_3$/1, range 0.2-7.1), acidity (potentiometric titration to pH 8.3; 6.9 mg $CaCO_3$/1, 2.5-16.4), calcium and hardness (EDTA titration; 3.5 mg/1, 1.8-6.7 and 8.0 mg $CaCO_3$/1, 4.0-14.5), chloride (mercuric nitrate titration; 20.2 mg/1, 16.8-22.6), sulfate (turbidimetric method; 7.4 mg/1, 2.5-10.7), and aluminum (eriochrome cyanine R method; 0.02 mg total Al/1, 0.005-0.03). The above parameters were measured according to standard methods (APHA, 1985).

Water from the same bog was pumped (ASM Industries, Inc., model S2B326 pump) to and stored in 160 liter plastic containers. Prior to use, it was filtered into 20 liter polyethylene carboys through Fisher P8 filter paper to remove suspended materials and then adjusted to the experimental temperature in the walk-in refrigerator. The experimental solutions were prepared a minimum of 24 hours prior to the initiation of each experiment. Aluminum concentrations were adjusted by adding the appropriate amount of solid $AlCl_3 \cdot 6H_2O$ to the filtered bog water. The pH was lowered using an artificial acid rain mixture consisting of 6.5 parts concentrated H_2SO_4 and 3.0 parts concentrated HNO_3 (Correa et al., 1985). When necessary, a solution of NaOH was used to raise the pH. Aluminum concentrations and pH were monitored at least bidaily and daily, respectively, using the previously described procedures. The mean measured pH and total aluminum

concentrations (mg/l) are listed in parentheses following the nominal values in each experimental section. Unless otherwise indicated, the solutions used in experiments with static exposures were renewed daily.

Toxicology

Acute Toxicity

Separate tests were performed to determine those levels where low pH and aluminum ceased to be acutely toxic. The pH levels (10) ranged from 6.1 (control) to 1.0 and varied less than 0.1 units from the nominal pH. Aluminum concentrations (5) ranged from 0.01 (control) to 500 mg total Al/l. In the latter assay, the pH was not adjusted back to the control level and was 4.1 or less (3.6 for 500 mg/l to 4.1 for 62.5 mg/l) in all tested concentrations.

Gravity feed flow-through systems (Figure 1), modified from Correa et al. (1983), were used at a flow rate of 5 ml/minute. This rate was found to be sufficient to maintain an outflow dissolved oxygen concentration of greater than 5 mg O_2/l. Ten nymphs were placed into each 250 ml test chamber for 96 hours. During the test, the nymphs were observed several times daily and any dead nymphs removed. A nymph was considered dead if it did not respond to gentle prodding.

The 96 hour LC50 for pH and its 95% confidence interval were determined employing the graphical method of Litchfield and Wilcoxon (1949). The mean hydrogen ion concentrations were plotted

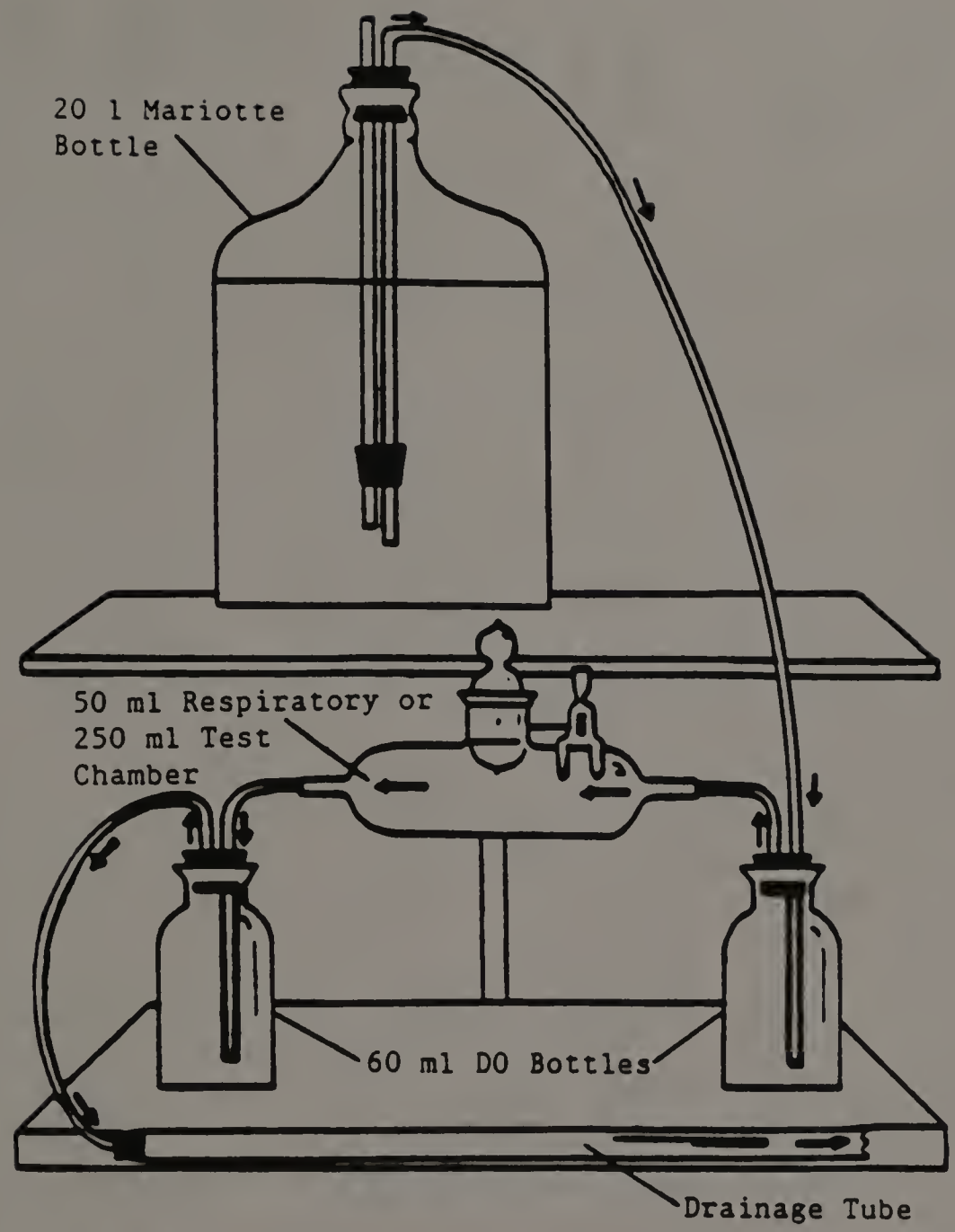

Figure 1. A gravity feed flow-through apparatus for toxicity testing and for measuring oxygen consumption of aquatic macroinvertebrates [modified from Correa et al. (1983)].

against percent mortality on logarithmic-probability paper. The results were converted to and reported in pH units. Whenever possible, median survival times were estimated by interpolating the time when 50% mortality occurred on a plot of survival time versus cumulative percent mortality on logarithmic-probability paper. Due to a lack of response at any test concentration, a LC50 for aluminum was not determined.

Subchronic Toxicity

Also pertinent to the research are the effects of long term exposure to elevated aluminum concentrations at low pH. Subchronic toxicity (90 days) was monitored at pH levels of 6.4, 5.0, 3.7, and 2.4 with added aluminum concentrations of 0, 0.5, and 5 mg/l. To further clarify the results, treatments of 10 and 30 mg Al/l at pH 2.4 and 10 mg/l at pH 5.0 were subsequently examined. In these experiments, total and soluble (sample filtered through 0.45 um membrane filter prior to analysis) aluminum concentrations were measured at least once weekly.

The nymphs were sexed using as the criterion a discoloration of the cuticle on the sternites of the 2nd and 3rd abdominal segments. These swellings indicate the position of the genital sac in adult male Libellulidae (Fraser, 1939, 1943; Westfall, 1984). Five nymphs of each sex were separately exposed to each treatment in glass jars containing 2.5 liters of adjusted water. Each jar was provided with fiberglass screening (extending above the water's surface) to which the nymphs could cling (or emerge). Mosquito

larvae (10/nymph) were offered once weekly and the test solutions renewed 2 to 3 times per week. During exposure, the nymphs were observed daily and mortality and any evidence of cannibalism recorded. Median survival times, their 95% confidence limits, and significant differences between the treatments were determined for each pH/aluminum combination (sexes pooled) using the nomographic method of Litchfield (1949) and Litchfield and Wilcoxon (1949). Nymphs that emerged (< 3%), attempted to emerge (incomplete ecdysis, < 3%), or showed extensive injuries due to cannibalism (< 2%) were excluded from analysis.

Physiology

Water Balance and Cation Body Burdens

Water balance and whole body cation regulation were examined in nymphs exposed to a control (5.75/0.009) and to test solutions at pH 2.3 with 0 (2.34/0.025) and 30 (2.34/29.0) mg/l added aluminum. Changes in wet weight were also examined at pH 2.3 with 0.3 (2.31/0.32) and 3 (2.31/3.14) mg Al/l and at pH 4.0 with 0 (3.99/0.019), 0.3 (4.01/0.30), 3 (4.02/2.99), and 30 (4.00/28.4) mg Al/l. Initial wet weights were determined by blotting each nymph for 1 minute and weighing to the nearest 0.1 mg on a Mettler H35AR balance. Following a 96 hour exposure in 300 ml DO bottles, the nymphs were blotted as before and their final wet weights determined. The nymphs were then rinsed, quickly killed in hot deionized water, and dried to a constant weight at 50 C (> 48

hours, dry weight) in a Boekel oven. They were then transferred to porcelain crucibles and ignited to a constant weight at 550 C (> 3 hours, ash weight) in a Lindberg box furnace. The ashed samples were digested in 1.00 ml of concentrated HNO_3 (Baker Instra-Analyzed acid), diluted to 10.0 ml with deionized water, and analyzed for Na^+, K^+, Ca^{2+}, and Mg^{2+} using a Perkin-Elmer ICP/6500 atomic emission spectrophotometer.

To determine the rate of wet weight loss, the specimens were weighed prior to and at 24 hour intervals during a 96 hour exposure to the first 3 treatments described above (5.93/0.009, 2.31/0.021, and 2.31/31.3).

Hemolymph Osmolality and Electrolyte Concentrations

In this series of experiments, the nymphs were exposed in glass jars containing 2.5 liters of test solution. Subsequently, each nymph was rinsed for 10 seconds in a stream of deionized water and blotted dry with paper. The hemolymph was collected from a small puncture made with forceps in the lateral portion of the intersegmental membrane between the 6th and 7th abdominal pleurites [laterosternites of Snodgrass (1954)] with a borosilicate glass capillary micropipet. Using this procedure, it was possible to collect in excess of 50 ul of hemolymph from control insects. The hemolymph was transferred to 400 ul polypropylene microcentrifuge tubes (on ice) and spun for 5 minutes in a Beckman microfuge (model 152) to remove hemocytes.

Changes in hemolymph osmolality were examined in nymphs exposed for 96 hours to a control (5.84/0.006) and test solutions at pH 2.3 and 4.0 with 0 (2.32/0.011, 3.97/0.011) and 30 (2.32/30.6, 3.96/30.7) mg/l added aluminum. The osmolality of an 8 ul sample of supernatant was measured with a Wescor vapor pressure osmometer (model 5100C).

Hemolymph Cl^- concentrations were measured in nymphs exposed for 96 hours to a control (5.90/0.006) and test solutions at pH 2.3 and 4.0 with 0 (2.31/0.014, 3.97/0.011) and 30 (2.32/31.3, 3.96/30.7) mg Al/l. Also examined was the response in a solution at pH 2.3 with 30 (2.31/33.7) mg Al/l added as the sulfate salt [$Al_2(SO_4)_3 \cdot 18H_2O$]. Cl^- concentrations were determined using the mercuric thiocyanate method of Florence and Farrar (1971) substituting nitric acid (uncolored) for perchloric acid. A suitable portion (2-4 ul) of supernatant was added to 20 ml of deionized water in a 25 ml volumetric flask. Following reagent addition, the samples were made up to volume and their absorbances read versus a reagent blank after 5 minutes at 460 nm using a Perkin-Elmer Hitachi 200 spectrophotometer with a 5 cm pathlength. Duplicate samples were prepared for each nymph and the mean value employed in all subsequent analyses.

Hemolymph cation concentrations were determined in nymphs exposed for 96 hours to a control (6.24/0.009) and test solutions at pH 2.3 and 4.0 with added aluminum concentrations of 0 (2.34/0.054, 4.02/0.022), 0.3 (2.34/0.34, 4.06/0.32), and 30 (2.33/31.2, 4.02/32.8) mg/l. An appropriate quantity (10-30 ul) of

supernatant was diluted to 10.0 ml with deionized water. These were analyzed for Na^+, K^+, Ca^{2+}, and Mg^{2+} using a Perkin-Elmer ICP/6500 atomic emission spectrophotometer.

In addition, the effect of a 192 hour exposure to a control (5.89/0.006) and test solutions at pH 2.3 with 0 (2.31/0.015), 0.3 (2.31/0.29), and 3 (2.31/2.94) mg/l added aluminum were investigated. In this case, 10 ul of supernatant were diluted to 2.0 ml with a 15 mmol/l lithium solution, and Na^+ and K^+ concentrations determined using a flame photometer (Instrumentation Laboratory, model IL443).

Hemolymph Volume

Hemolymph volume was estimated using a modification of the isotopic dilution technique of Loughton and Tobe (1969). Following a 96 hour exposure to the test solutions [control (5.81/0.011) and pH 2.3 with 0 (2.31/0.022) and 30 (2.30/31.4) mg/l added aluminum], each nymph, rinsed for 10 seconds in a stream of deionized water and blotted dry, was injected with 5.0 ul of a saturated solution of ^{14}C-carboxy-inulin (Sigma Chemical Co., specific activity 2.6 mCi/g) dissolved in deionized water. The needle was inserted into the thorax through a foreleg previously amputated at the distal end of the femur (Gringorten and Friend, 1979). A ligature was tied at the distal end of the femur to prevent leakage of hemolymph and tracer. The nymph was then returned to a tube containing the appropriate test solution for 60 minutes to allow the inulin to distribute throughout the hemocoel. The nymph was rinsed and

blotted, and the hemolymph collected and centrifuged as described earlier. Duplicate 5.0 ul aliquots of the supernatant from each insect were transferred to borosilicate glass tubes, 450 ul of Aquasol-2 (New England Nuclear Co.) added to each, and the tubes vortexed. Each tube was placed into a scintillation vial and counted twice (2 minutes or a minimum of 5000 counts) in a Packard Tri-Carb liquid scintillation spectrophotometer (model 3255). Aliquots of supernatant without tracer (background) and with a known volume of tracer (to determine counts injected) were also prepared and counted as describe above.

The total extracellular fluid volume (i.e., hemolymph volume) was calculated as:

$$V_H = [(cpm_i - cpm_b)(V_a)/(cpm_a - cpm_b)] - V_i$$

where,

V_H = total hemolymph volume in ul

V_a = volume of hemolymph aliquot counted (5.0 ul)

V_i = volume of inulin solution injected (5.0 ul)

cpm_i = total radioactivity injected (174,000 counts per minute)

cpm_b = background radioactivity

cpm_a = total radioactivity of hemolymph aliquot.

The mean of the four values determined for each insect was used in all subsequent analyses.

Acid-Base Balance -- Hemolymph pH

Fluctuations in hemolymph pH were measured in nymphs exposed in flow-through systems to a control (6.18/0.008) and test solutions

at pH 2.3 and 4.0 with 0 (2.31/0.020, 3.99/0.019), 0.3 (2.31/0.32, 4.01/0.30), 3 (2.31/3.14, 4.02/2.99), and 30 (2.31/30.7, 4.00/28.4) mg/l added aluminum. Following a 96 hour exposure, each nymph was rinsed for 10 seconds in a stream of deionized water and blotted dry with paper. The nymph was then immobilized on its back with pins in a wax lined petri dish. Using forceps, a 2-3 mm tear was made in the lateral portion of the intersegmental membrane between the 6th and 7th abdominal laterosternites to accommodate insertion of the microcombination pH probe (Microelectrodes, Inc., MI-410). The pH was recorded on a Beckman pH meter (model 70).

Oxygen Consumption

During each of four runs, 5 nymphs were exposed, 1 per treatment, to a control (6.08/0.009) and test solutions at pH 4.0 with added aluminum concentrations of 0 (3.99/0.019), 0.3 (4.01/0.30), 3 (4.02/2.99), and 30 (4.00/28.4) mg/l. Oxygen consumption was measured using flow-through systems (Figure 1) which provided test water at a rate of 5 $\pm$ 0.5 ml/minute. Following the determination of initial wet weights, the nymphs were placed individually into the 50 ml respiratory chambers and oxygen uptake determined after 24, 40, 48, 64, 72, 88, and 96 hours. The dissolved oxygen concentrations in the 60 ml DO bottles upstream and downstream of each respiratory chamber were measured using the Alsterberg azide modification of the Winkler method (APHA, 1985). Respiration was calculated as:

$$R = 60(F \times D)/W$$

where,

R = oxygen consumption in ug O_2/hr·g initial wet weight

F = flow rate of the water through the system in ml/minute

D = dissolved oxygen differential between the upstream and downstream bottles in mg O_2/l

W = initial wet weight of nymph in grams

60 = constant converting minutes to hours, ml to l, and mg to ug.

Data Treatment

Statistical analyses (analysis of variance or covariance) were performed on the University of Massachusetts´ mainframe computer (Cyber 175, Control Data Corporation) using either the BMDP-87 statistical software package (Dixon et al., 1983) or the mixed model least-squares and maximum likelihood (MMLSML) computer program (Harvey, 1985). Results of the oxygen consumption study were analyzed by means of a randomized block, split-plot design (MMLSML, model 6). One-way analyses of covariance (MMLSML, model 1) were used to determine significance in the wet, dry, and ash weight experiments. In each of these analyses, initial wet weight was employed as the covariate. An analysis of covariance for a completely randomized, split-plot design (BMDP2V) was adopted to analyze the rate of wet weight loss experiment. As before, the initial wet weight was used as the covariate. The results of the remaining parameters were tested utilizing individual one-way analyses of variance (BMDP2D or MMLSML, model 1). When

statistically significant differences ($P < 0.05$) were indicated in the analysis of variance or covariance, Duncan´s new multiple range test (Damon and Harvey, 1987) was used to determine which treatment means (or adjusted treatment means for covariance) differed significantly ($P < 0.05$) from the rest.

CHAPTER IV

RESULTS

Toxicology

Acute Toxicity

The 96 hour LC50 for pH and its 95% confidence limits were determined to be 2.01 (1.88-2.14). The lowest test pH where no acute toxicity was observed was 2.3. Median survival times were estimated to be 21 hours at pH 1.0, 33 hours at pH 1.5, and 64 hours at pH 1.9.

In the acute aluminum toxicity test, no mortality could be attributed to aluminum at concentrations as high as 500 mg/l (at pH 3.6), even during an extended exposure period of 11 days.

Subchronic Toxicity

The results of the subchronic toxicity tests as well as the nominal and measured pH and aluminum levels used are shown in Table 1. Of the 15 pH/aluminum combinations tested, only two (6.4/0.5 and 2.4/5) showed indications of shorter survival times in one sex relative to the other. In both of these treatments, all of the reacting female nymphs succumbed prior to, or at the same time as, the first reacting male nymph. Because of this (only 2 of 15 showed differences) and the fact that only 5 nymphs per sex were exposed to each treatment, the results for each treatment were pooled prior to analysis.

At 90 days, survival by nymphs in the control (pH 6.4, 0 mg/l added aluminum) exceeded 50%; therefore, it was not possible to establish a median survival time with a 95% confidence interval. Because of this, comparisons between the control and each treatment could not be made. Thus, the only comparisons between median survival times made were those among the aluminum concentrations within each pH and those among the pHs within each nominal aluminum concentration (Figure 2).

At pH 2.4, survival times for nymphs exposed to 0, 0.5, and 5 mg Al/l were significantly ($P < 0.05$) longer than those found in the 10 and 30 mg/l treatments. The median survival time observed at pH 5.0 with 10 mg/l added aluminum was significantly shorter than that found in the 0 mg/l treatment, but not shorter than those of the 0.5 and 5 mg/l treatments. Within the pH 3.7 and 6.4 treatments, no significant effects of aluminum on survival were observed. Within the 0, 0.5, and 5 mg Al/l treatments, survival times at pH 2.4 were found to be significantly shorter than those observed at pH 3.7, 5.0, and 6.4. Furthermore, nymphs in the 2.4/10 treatment had significantly shorter survival times than those in the 10 mg/l added aluminum treatment at pH 5.0 (3.7/10 and 6.4/10 not examined). Exposure to the 3.7/5 treatment reduced the survival time significantly compared with the 6.4/5, but not the 5.0/5 treatment.

Physiology

Water Balance and Cation Body Burdens

Following a 96 hour exposure to the test solutions, highly significant differences ($P < 0.0001$) in wet weight were found to exist among the treatments. Specifically, the wet weights of nymphs exposed to the 2.3/0 and 2.3/30 treatments were found to be significantly ($P < 0.05$) lower than those of the control (Table 2). Neither the 2.3/0.3, 2.3/3, nor the any of the treatments at pH 4.0 elicited a response significantly different from the control. In addition, the wet weights of nymphs exposed to the 2.3/30 treatment were found to be significantly lower than those observed in any of the other treatments.

In the initial analysis of covariance for the wet weight loss through time experiment, highly significant differences ($P < 0.0001$) were found to exist among the treatments and times employed, as well as for the interaction between these effects. Because of this interaction, the treatments were examined at each time using individual analyses of covariance. When significant, Duncan´s new multiple range test was used to separate the treatment means. During the first 24 to 48 hours, there was a small wet weight gain by nymphs in all of the treatments (including the control). This was followed by a substantial loss of wet weight during the latter portion of the exposure period by nymphs in the 2.3/0 and 2.3/30 treatments. These losses were found to be

significant ($P < 0.05$) compared with the control (and each other) at 72 and 96 hours (Figure 3).

The adjusted mean dry and ash weights are presented in Table 3. In the analysis of covariance, no significant differences ($P > 0.05$) in dry weight were found to exist among the treatments. For ash weight, however, highly significant differences ($P < 0.0001$) among the treatments were found to exist. Relative to the control, significantly ($P < 0.05$) lower ash weights were observed in nymphs exposed to the 2.3/0 and 2.3/30 treatments. Furthermore, the reduction found in the 2.3/30 treatment was significantly greater than that observed in the 2.3/0 treatment.

In separate analyses of variance, highly significant differences were found among the treatments for body burdens of Na^+ ($P < 0.0001$) and Ca^{2+} ($P < 0.01$). The mean body burden of Na^+ in nymphs exposed to the 2.3/30 treatment was significantly ($P < 0.05$) lower than those observed in nymphs from the control and 2.3/0 treatment. No significant differences were found between the latter two values (Table 4). A significantly lower body burden of Ca^{2+} was observed in nymphs exposed to the 2.3/30 treatment relative to the 2.3/0 treatment, although neither differed significantly from the control (Table 5). Statistical analyses of the body burdens of K^+ and Mg^{2+} did not reveal any differences among the treatments (Tables 4 and 5).

Hemolymph Osmolality and Electrolyte Concentrations

Following a 96 hour exposure, highly significant differences ($P < 0.0001$) in hemolymph osmolality as well as hemolymph Cl^- and Na^+ concentrations were found to exist among the treatments. Although slightly elevated, none of the tested pH 4.0 treatments provoked a response significantly different ($P > 0.05$) from their respective control values for these three parameters. Differences were observed, however, for the pH 2.3 treatments. Namely, significantly reduced osmolalities relative to the control (and pH 4.0 treatments) were observed in nymphs exposed to the 2.3/0 and 2.3/30 treatments. In addition, the reduction observed in the 2.3/30 treatment was significantly greater than that caused by the 2.3/0 treatment (Table 6). In contrast, hemolymph Cl^- (Table 7) and Na^+ (Table 8) concentrations significantly lower than those found in the controls (and all other treatments) were observed only in the 2.3/30 treatments. Although the 2.3/0 treatment values were depressed, they were not significantly lower than those of the control. At pH 2.3, 30 mg/l of aluminum added as the chloride salt ($AlCl_3 \cdot 6H_2O$) caused a greater, but not significantly greater, reduction in hemolymph Cl^- levels than 30 mg Al/l added as the sulfate salt [$Al_2(SO_4)_3 \cdot 18H_2O$].

Hemolymph concentrations of K^+, Ca^{2+}, and Mg^{2+} are shown in Tables 8 and 9. No significant differences ($P > 0.05$) among the treatments were found for any of these cations following a 96 hour exposure to the various test solutions.

In contrast to the above results, a 192 hour exposure to test solutions at pH 2.3 with 0, 0.3, and 3 mg/l added aluminum provoked highly significant differences in the hemolymph concentrations of both Na^+ ($P < 0.0001$) and K^+ ($P < 0.01$). Hemolymph Na^+ concentrations in nymphs exposed to these three treatments were significantly ($P < 0.05$) reduced while K^+ concentrations were significantly elevated relative to their respective controls (Table 10). For both cations, however, no significant differences were observed among the three treatments (aluminum concentrations) at pH 2.3.

Hemolymph Volume

Highly significant differences ($P < 0.001$) in hemolymph volume were found to exist among the treatments. Specifically, values from nymphs exposed to the 2.3/30 treatment were significantly ($P < 0.05$) lower than those found in the control and the 2.3/0 treatment. Although a volume reduction was observed in the 2.3/0 treatment, it was not significant compared to the control (Table 11).

Acid-Base Balance -- Hemolymph pH

Highly significant differences ($P < 0.0001$) were found to exist for hemolymph pH among the treatments. Significantly ($P < 0.05$) depressed pH values relative to the control were observed in the 4.0/30 treatment as well as in all of the treatments at pH 2.3 (0, 0.3, 3, and 30 mg Al/l). The hemolymph pH in nymphs exposed to the

pH 4.0 treatments with 0, 0.3, and 3 mg Al/l did not differ significantly from the control. In addition, pH values observed for nymphs in the 2.3/30 treatment were significantly lower than those found in all of the other treatments (Table 12).

Oxygen Consumption

As a consequence of elevated hydrogen ion and aluminum concentrations, highly significantly ($P < 0.01$) depressed oxygen consumption was observed among the treatments (Figure 4). Although the mean oxygen uptake was reduced by 28% in the 4.0/0 treatment and by 36% in the 4.0/0.3 treatment compared to control rates, these differences were not significant ($P > 0.05$). Reductions significantly different from the control were observed, however, at pH 4.0 with added aluminum concentrations of 3 and 30 mg/l (54% and 75%, respectively). Furthermore, respiration rates of nymphs in the 4.0/30 treatment were significantly lower than those observed in both the 4.0/0 and 4.0/0.3 treatments. Low pH (4.0) alone, therefore, does not appear to inhibit oxygen uptake as markedly as when used in conjunction with aluminum in concentrations of 3 mg/l and above.

The effect of aluminum at low pH through time for the individual treatments is shown in Figure 5. No significant differences ($P > 0.05$) in oxygen consumption were found to exist among the times used in this experiment. Although not measured within the first 24 hours of exposure, it is apparent that both aluminum and low pH impair respiration within this time frame.

Table 1. Median survival times with 95% confidence limits and percent survival values following a 90 day exposure to aluminum at low pH.

Al added (mg/l)	pH Mean	pH Range	Average Measured Al Concentration (mg/l) Total	Soluble	Median Survival Time with 95% Confidence Limits		Percent Survival at Day 90
0.0	2.40	2.38-2.44	0.03	—	9.8	(8.2-11.8)	0
0.5	2.40	2.37-2.43	0.50	—	11.0	(9.6-12.7)	0
5.0	2.38	2.35-2.43	5.13	—	10.5	(9.2-12.0)	0
10.0	2.40	2.35-2.43	11.2	—	7.0	(5.4-9.1)	0
30.0	2.41	2.37-2.45	30.6	—	5.4	(3.6-8.0)	0
0.0	3.73	3.66-3.78	0.02	0.02	56	(42-74)	22
0.5	3.74	3.67-3.80	0.55	0.54	58	(43-78)	33
5.0	3.73	3.68-3.77	4.74	4.66	39	(28-54)	22
0.0	5.08	4.90-5.28	0.008	0.007	73	(58-92)	33
0.5	5.09	4.95-5.31	0.27	0.15	67	(34-131)	44
5.0	4.91	4.75-5.04	2.39	1.66	62	(43-89)	33
10.0	4.98	4.83-5.15	3.63	2.46	46	(32-66)	20
0.0	6.43	6.24-6.69	0.005	0.005	—		62
0.5	6.45	6.27-6.61	0.14	0.09	82	(41-164)	44
5.0	6.46	6.31-6.69	1.24	0.29	63	(47-84)	22

— not determined

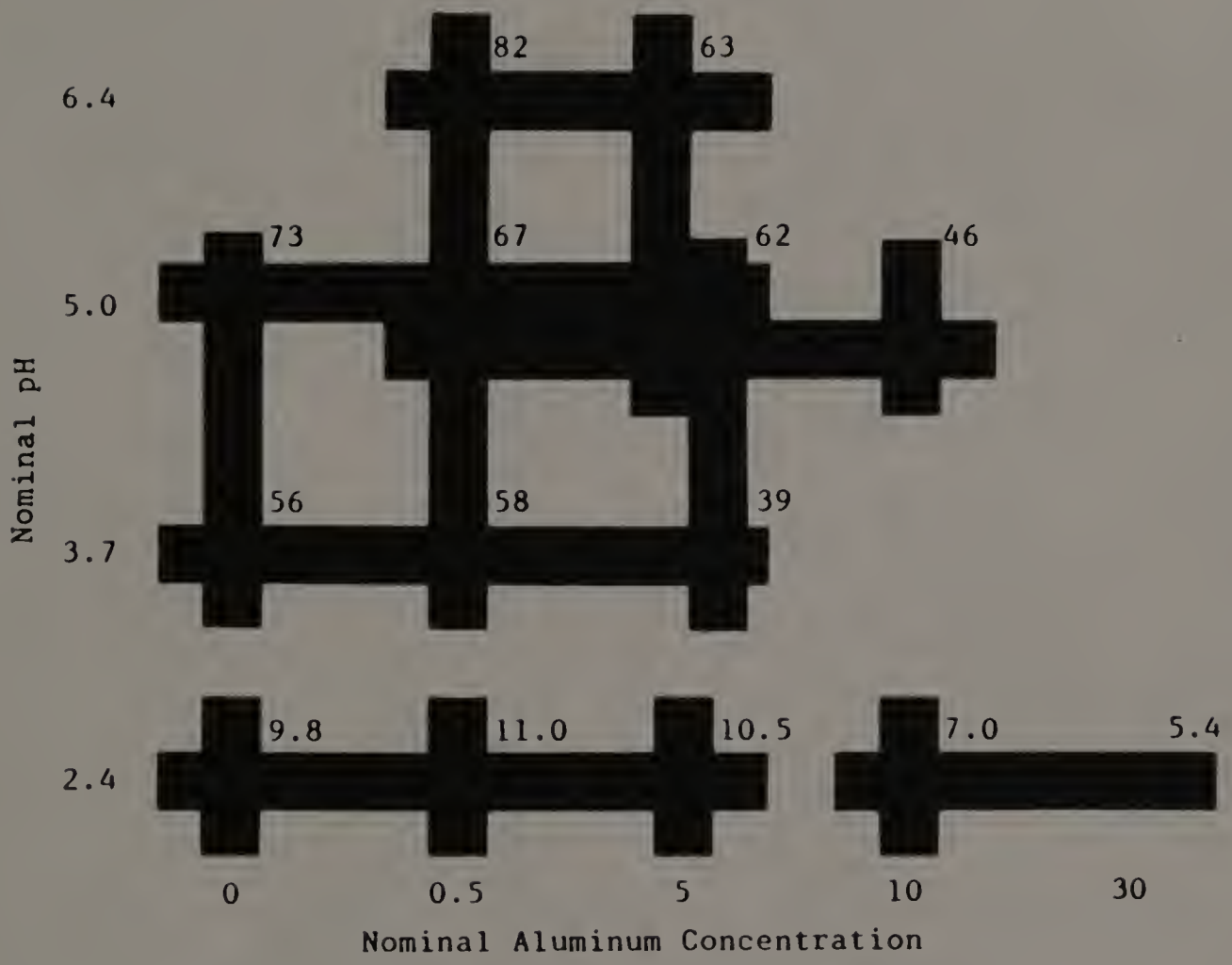

Figure 2. Statistical analysis of median survival times for nymphs exposed to aluminum at low pH for 90 days. The numbers within the figure are the median survival times for the tested pH/aluminum combinations. Within each nominal pH and each nominal aluminum concentration (mg/l), any two values not connected by a bar are significantly different ($P < 0.05$) and any two values connected by a bar are not significantly different.

Table 2. Changes in wet weight following exposure to aluminum at low pH for 96 hours. Adjusted least square means in mg ± 1.96 S.E.

Treatment	n	Wet Weight
Control	19	309.2 ± 3.02 ab
4.0/0	5	309.0 ± 5.86 abc
4.0/0.3	4	306.2 ± 6.55 abc
4.0/3	5	315.5 ± 6.06 a
4.0/30	5	313.4 ± 5.86 a
2.3/0	13	301.6 ± 3.65 c
2.3/0.3	6	302.5 ± 5.51 bc
2.3/3	6	302.9 ± 5.37 bc
2.3/30	14	278.8 ± 3.49 d

Note: Any two means with no letters in common differ significantly ($P < 0.05$, Duncan's new multiple range test).

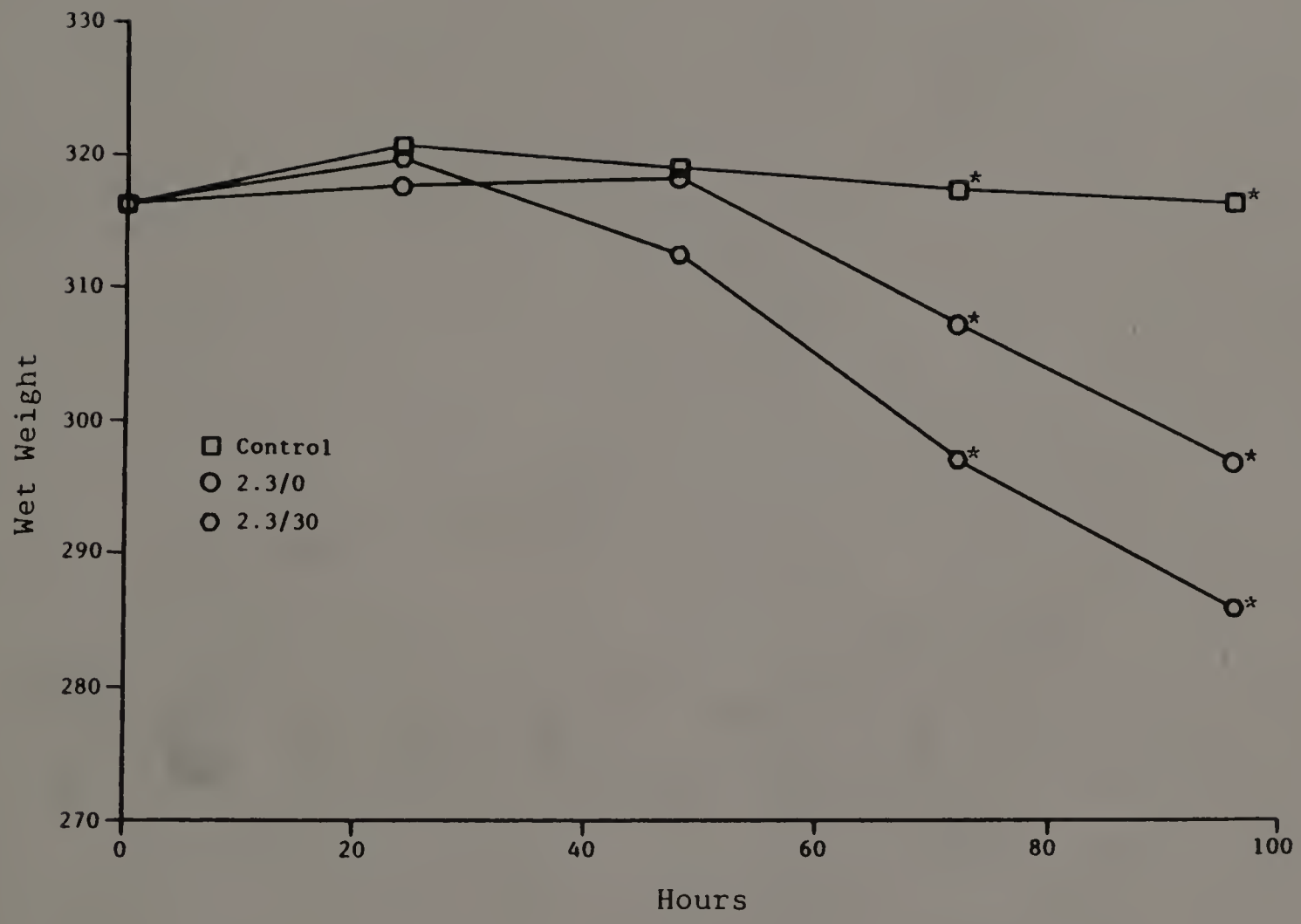

Figure 3. Wet weight during a 96 hour exposure to aluminum at low pH. Points are adjusted least square means in mg of 9-14 observations. The legend shows nominal pH/Al (mg/l) levels. * indicates significant difference ($P < 0.05$) from all other treatments at this time period.

Table 3. Changes in dry and ash weights following exposure to aluminum at low pH for 96 hours. Adjusted least square means in mg ± 1.96 S.E.

Treatment	n	Dry Weight	Ash Weight
Control	8	62.0 ± 2.78	3.1 ± 0.14 a
2.3/0	7	57.9 ± 3.06	2.7 ± 0.16 b
2.3/30	8	59.7 ± 2.82	2.3 ± 0.14 c

Note: For ash weight, any two means with no letters in common differ significantly ($P < 0.05$, Duncan's new multiple range test).

Table 4. Changes in the body burdens of sodium and potassium following exposure to aluminum at low pH for 96 hours. Means in ug/insect ± 1.96 S.E.M.

Treatment	n	Sodium	Potassium
Control	8	489 ± 34.7 a	322 ± 34.1
2.3/0	7	487 ± 52.9 a	310 ± 33.5
2.3/30	8	189 ± 43.5 b	333 ± 33.1

Note: For sodium, any two means with no letters in common differ significantly ($P < 0.05$, Duncan's new multiple range test).

Table 5. Changes in the body burdens of calcium and magnesium following exposure to aluminum at low pH for 96 hours. Means in ug/insect ± 1.96 S.E.M.

Treatment	n	Calcium	Magnesium
Control	8	52 ± 4.7 ab	52 ± 3.1
2.3/0	7	58 ± 5.3 a	55 ± 5.1
2.3/30	8	47 ± 3.5 b	52 ± 4.9

Note: For calcium, any two means with no letters in common differ significantly ($P < 0.05$, Duncan's new multiple range test).

Table 6. Changes in hemolymph osmolality following exposure to aluminum at low pH for 96 hours. Means in mmol/kg ± 1.96 S.E.M.

Treatment	n	Osmolality
Control	10	303 ± 6.1 a
4.0/0	6	306 ± 12.5 a
4.0/30	6	304 ± 9.0 a
2.3/0	9	260 ± 16.1 b
2.3/30	6	220 ± 8.4 c

Note: Any two means with no letters in common differ significantly ($P < 0.05$, Duncan's new multiple range test).

Table 7. Changes in hemolymph chloride concentration following exposure to aluminum at low pH for 96 hours. Means in mmol/l ± 1.96 S.E.M.

Treatment	n	Chloride
Control	19	129 ± 5.7 a
4.0/0	6	134 ± 16.7 a
4.0/30	6	133 ± 6.7 a
2.3/0	15	117 ± 9.0 a
2.3/30	9	93 ± 9.6 b
2.3/30*	15	102 ± 12.5 b

* sulfate salt

Note: Any two means with no letters in common differ significantly ($P < 0.05$, Duncan's new multiple range test).

Table 8. Changes in hemolymph sodium and potassium concentrations following exposure to aluminum at low pH for 96 hours. Means in mmol/l ± 1.96 S.E.M.

Treatment	n	Sodium	Potassium
Control	5	153 ± 10.2 a	3.3 ± 0.41
4.0/0	6	156 ± 4.9 a	3.6 ± 0.57
4.0/0.3	5	154 ± 4.7 a	3.6 ± 0.49
4.0/30	6	159 ± 5.1 a	3.7 ± 0.33
2.3/0	5	142 ± 21.0 a	4.4 ± 0.49
2.3/0.3	6	153 ± 8.2 a	3.2 ± 0.59
2.3/30	4	104 ± 16.7 b	4.1 ± 1.71

Note: For sodium, any two means with no letters in common differ significantly ($P < 0.05$, Duncan's new multiple range test).

Table 9. Changes in hemolymph calcium and magnesium concentrations following exposure to aluminum at low pH for 96 hours. Means in mmol/l ± 1.96 S.E.M.

Treatment	n	Calcium	Magnesium
Control	5	4.5 ± 1.00	6.1 ± 0.78
4.0/0	6	5.8 ± 0.53	6.1 ± 0.43
4.0/0.3	5	4.6 ± 1.27	6.7 ± 0.78
4.0/30	6	5.3 ± 0.67	6.1 ± 1.00
2.3/0	5	4.6 ± 1.43	6.8 ± 1.25
2.3/0.3	6	5.5 ± 0.69	6.9 ± 0.82
2.3/30	4	5.1 ± 0.98	8.0 ± 1.78

Table 10. Changes in hemolymph sodium and potassium concentrations following exposure to aluminum at low pH for 192 hours. Means in mmol/l ± 1.96 S.E.M.

Treatment	n	Sodium	Potassium
Control	9	146 ± 3.5 a	4.0 ± 0.59 b
2.3/0	5	60 ± 11.6 b	11.2 ± 6.12 a
2.3/0.3	5	57 ± 11.0 b	12.4 ± 4.55 a
2.3/3	6	58 ± 6.3 b	9.6 ± 2.18 a

Note: In each column, any two means with no letters in common differ significantly ($P < 0.05$, Duncan's new multiple range test).

Table 11. Changes in hemolymph volume following exposure to aluminum at low pH for 96 hours. Means in ul ± 1.96 S.E.M.

Treatment	n	Volume
Control	5	150 ± 8.4 a
2.3/0	6	138 ± 17.1 a
2.3/30	4	87 ± 24.7 b

Note: Any two means with no letters in common differ significantly ($P < 0.05$, Duncan's new multiple range test).

Table 12. Changes in hemolymph pH following exposure to aluminum at low pH for 96 hours. Means ± 1.96 S.E.M.

Treatment	n	pH
Control	11	7.58 ± 0.074 a
4.0/0	5	7.53 ± 0.110 ab
4.0/0.3	4	7.48 ± 0.171 ab
4.0/3	5	7.57 ± 0.114 ab
4.0/30	4	7.36 ± 0.069 b
2.3/0	6	7.41 ± 0.090 b
2.3/0.3	6	7.38 ± 0.129 b
2.3/3	6	7.38 ± 0.100 b
2.3/30	5	6.77 ± 0.218 c

Note: Any two means with no letters in common differ significantly ($P < 0.05$, Duncan´s new multiple range test).

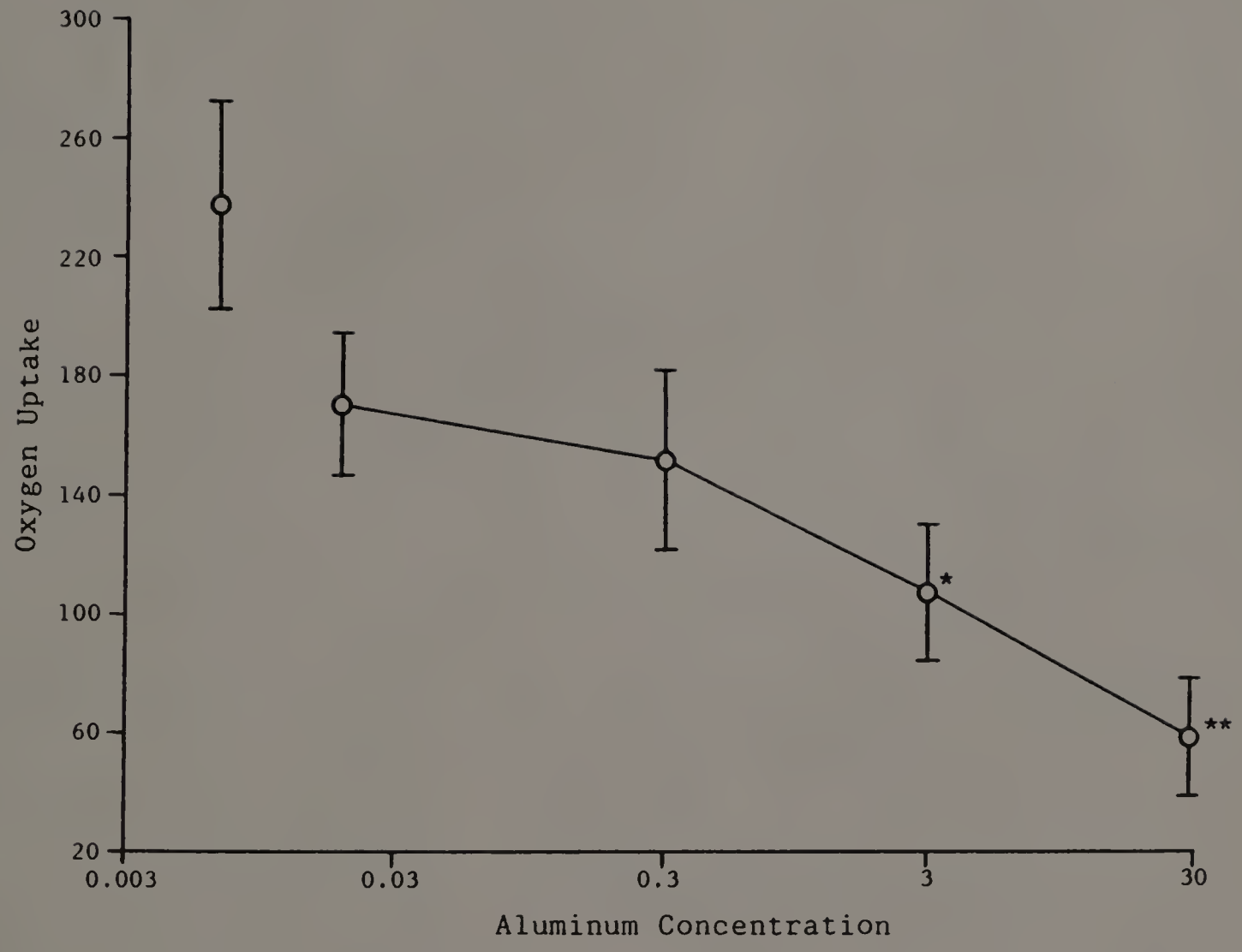

Figure 4. Oxygen uptake during a 96 hour exposure to various aluminum concentrations (mg/l) at pH 4.0. Control is shown for comparison. Points are means in ug O_2/h·g initial wet weight $\pm$ 1.96 S.E.M. of 26-28 observations. * indicates significant difference ($P < 0.05$) from control; ** indicates significant difference from control, 4.0/0, and 4.0/0.3 treatments.

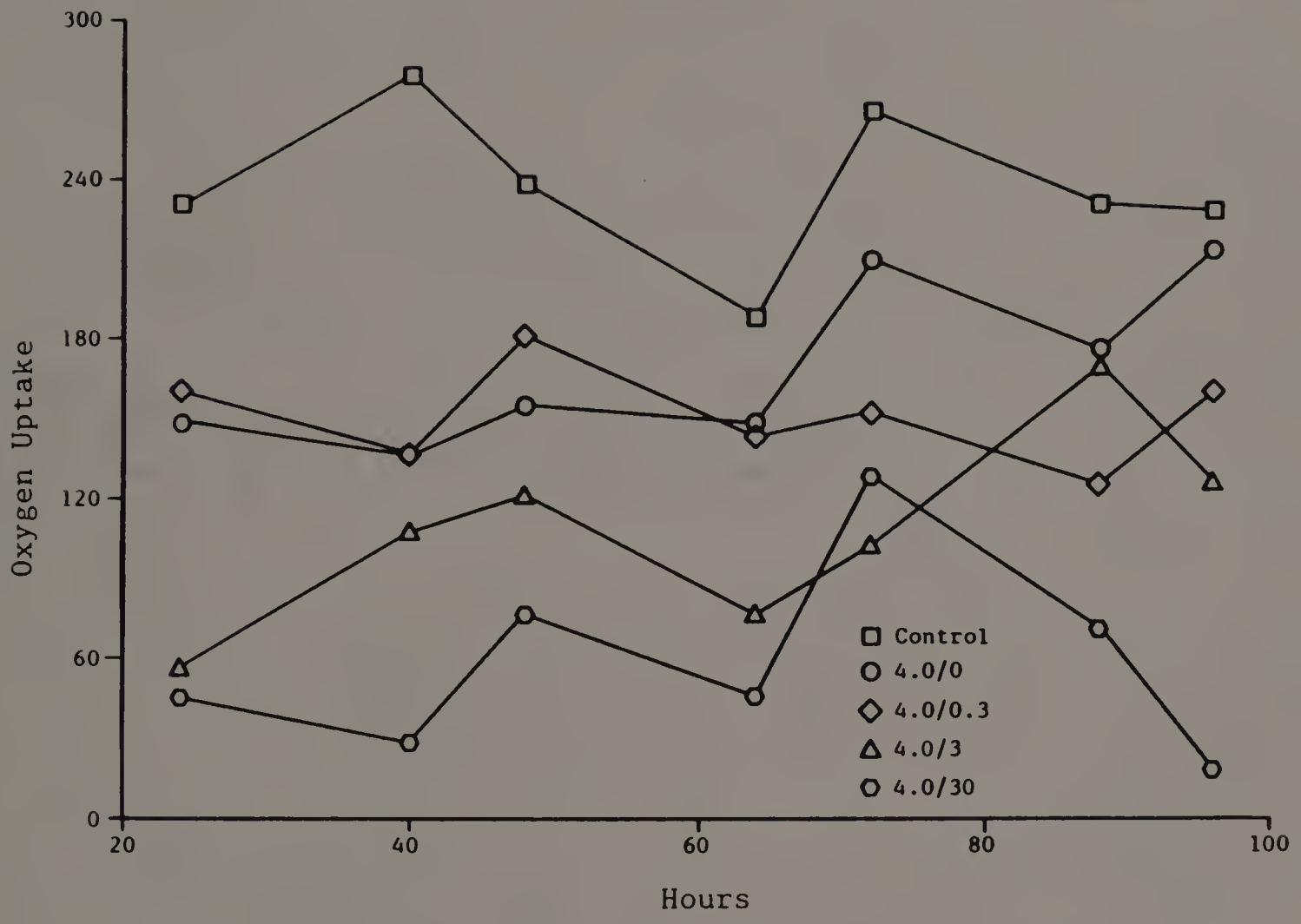

Figure 5. Oxygen uptake by nymphs in the individual treatments during a 96 hour exposure to aluminum at pH 4.0. Points are means in ug O_2/h·g initial wet weight of 3-4 observations. The legend indicates nominal pH/Al (mg/l) levels.

CHAPTER V

DISCUSSION

Toxicology

Acute Toxicity

In general, aquatic insects are tolerant of short term exposures to acidic conditions. In this study, the 96 hour LC50 for last instar nymphs of Libellula julia was determined to be pH 2.01. This result was not unexpected, however, because this species often occurs in abundance in the acidic waters of the Canadian Shield (Walker and Corbet, 1975). Similar toxicological results were obtained for the dragonfly Somatochlora cingulata and the damselfly Enallagma sp., where 96 hour LC50s for pH of 2.4 and 2.2 were recorded, respectively (Cruz, 1984; Mackie, 1989). Furthermore, Bell and Nebeker (1969) reported 96 hour LC50s of 3.25 and 3.5 for the moderately acid tolerant dragonflies Boyeria vinosa and Ophiogomphus rupinsulensis, respectively. In addition, nymphs of the dragonfly Libellula pulchella were observed to survive for greater than 12.5 hours at pH 1 (Stickney, 1922). In this regard, the median survival time for L. julia at pH 1.0 was found to be 21 hours with no mortality evident until 16 hours.

The effect of aluminum on aquatic organisms is highly variable. Fish, for instance, are unable to tolerate aluminum concentrations of 0.1 to 0.2 mg/l (Muniz and Leivestad, 1980a; Baker and

Schofield, 1982), while invertebrates, on the other hand, appear to be unaffected by higher, often much higher, concentrations. In this study, no acute mortality attributable to aluminum was observed in L. julia, even during an extended 11 day exposure to 500 mg Al/l at pH 3.6. A similar tolerance, as indicated by a 96 hour LC50 of 140 mg/l (test pH not reported), was found for the dragonfly Somatochlora cingulata (Cruz, 1984). At pH 3.5, 1 mg Al/l was not observed to be acutely toxic to the damselfly Enallagma sp. (Mackie, 1989), while 20 mg/l at pH 4.5 resulted in 100% mortality of Daphnia middendorffiana within 20 hours (Havas and Hutchinson, 1982). Although invertebrates as a group appear to be very tolerant of elevated aluminum levels, the importance of sublethal, chronic effects must not be ignored.

Subchronic Toxicity

In L. julia, lower median survival times were generally found with decreasing pH and with increasing concentrations of aluminum (Table 1, Figure 2). Differences among the median survival times were significant only when the pH was very low (2.4) or when the aluminum level was extremely high (nominal levels of 10 mg/l or more). At pH 2.4 with aluminum concentrations less than or equal to 5 mg/l and at pH 3.7 with 0.5 mg/l, additional toxicity due to aluminum, if it existed, was masked by the hydrogen ion toxicity. No significant mitigation of hydrogen ion toxicity by aluminum was observed at any of the tested pHs.

Similar trends have been reported for a number of aquatic insect species. Burton and Allan (1986) observed significantly higher mortality compared to the control (pH 7.0) in larvae of the stonefly Nemoura sp. and the caddisflies Pycnopsyche guttifer and Lepidostoma liba exposed for 28 days to pH 4.0 with and without added aluminum. Under some experimental conditions (e.g., low organic matter), 500 ug Al/l at pH 4.0 resulted in significant additional mortality for the former two species compared to pH 4.0 alone. No significant differences in survival for any of these species were found among the control and the pH 5.0 treatments with and without 250 ug Al/l.

In contrast, Herrmann (1987a) reported that the presence of aluminum (0.5 and particularly 2.0 mg inorganic Al/l) seemed to mitigate the detrimental impact of hydrogen ion stress in the mayflies Heptagenia sulphurea, Ephemera danica, Baetis rhodani, and Leptophlebia marginata exposed for 2 to 3 weeks at pH 4. The response of the more acid tolerant Heptagenia fuscogrisea to aluminum at pH 4 was not clear. With the exception of H. fuscogrisea, the mortality of these mayflies in the absence of aluminum was significantly higher at pH 4 than at pH 5. In addition, at pH 5 the beneficial effect of aluminum observed at pH 4 was reduced or reversed, resulting in increased mortality.

Havas and Hutchinson (1982) noted that the midges Chironomus riparius and Orthocladius consobrinus could survive exposure to pH 3.5 for periods of 50 hours to 20 days. Although mortality was evident, individuals of the caddisfly Limnephilus pallens

survived at this pH until the experiment was terminated after 38 days.

Bell (1971) found the period around emergence to be the most critical with respect to pH toxicity. The 30 day LC50s of nine stream insects ranged from 2.45 for the caddisfly Brachycentrus americanus to 5.38 for the mayfly Ephemerella subvaria. The 30 day LC50 values for these nine species were between 0.23 and 1.17 pH units higher than their respective 96 hour LC50s (Bell and Nebeker, 1969). The pHs required for 50% successful emergence ranged from 4.0 for B. americanus to 6.6 for the stonefly Isogenus frontalis and were between 0.52 and 2.1 units higher than the values obtained for their 30 day LC50s. For the dragonflies Ophiogomphus rupinsulensis and Boyeria vinosa, the 30 day LC50s were found to be 4.30 and 4.42, respectively. In both species, pH 5.2 was found to reduce successful emergence by 50%.

Physiology

Water Balance and Ionic Regulation

The procedure used to determine the wet weight of L. julia (i.e., blotting for 1 minute) was evaluated by weighing each of three nymphs four times. This method yielded results similar (standard errors of less than 0.2%) to those of the more elaborate procedure of Moens (1973). Thus, provided care was taken to see that the water was expelled from the rectum prior to each

measurement (Sayle, 1928; Dunson, 1980), this method proved to be extremely accurate.

In control nymphs, the hemolymph (assuming 1 ul = 1 mg) accounted for roughly 48% (150 ul/314.5 mg) of the wet weight. This compares favorably with the value (56% of 70 to 110 mg wet weight) reported for larvae of the alderfly Sialis lutaria (Beadle and Shaw, 1950; Shaw, 1955), but is considerably higher than the 32% and 34% found for the dragonflies Aeshna cyanea and Anax imperator, respectively (Moens, 1975a). Thus, in the smaller species, a greater proportion of the wet weight was accounted for by the hemolymph. The regression of hemolymph volume on wet weight for the latter two species (volumes determined by dye dilution method) and L. julia is shown in Figure 6.

The hemolymph composition of control nymphs (Tables 6 to 10) was consistent with the established pattern for this order (Sutcliffe, 1962, 1963; Bedford and Leader, 1975; Moens, 1975b; Dunson, 1980; Nicholls, 1983, 1985; Tembhare, 1984; Herzog, 1987). In category I hemolymph, the type characteristic of Odonata, Ephemeroptera, Plecoptera, Dictyoptera, and Hemiptera-Heteroptera, Na^+ and Cl^- account for the majority of the osmotic pressure, with K^+, Ca^{2+}, and Mg^{2+} making only minor contributions (Sutcliffe, 1963). Starvation for 10 days in pond or tap water did not markedly influence the hemolymph ionic composition of the dragonflies Aeshna cyanea and Libellula quadrimaculata (Moens, 1975b; Herzog, 1987; Nicholls, 1983).

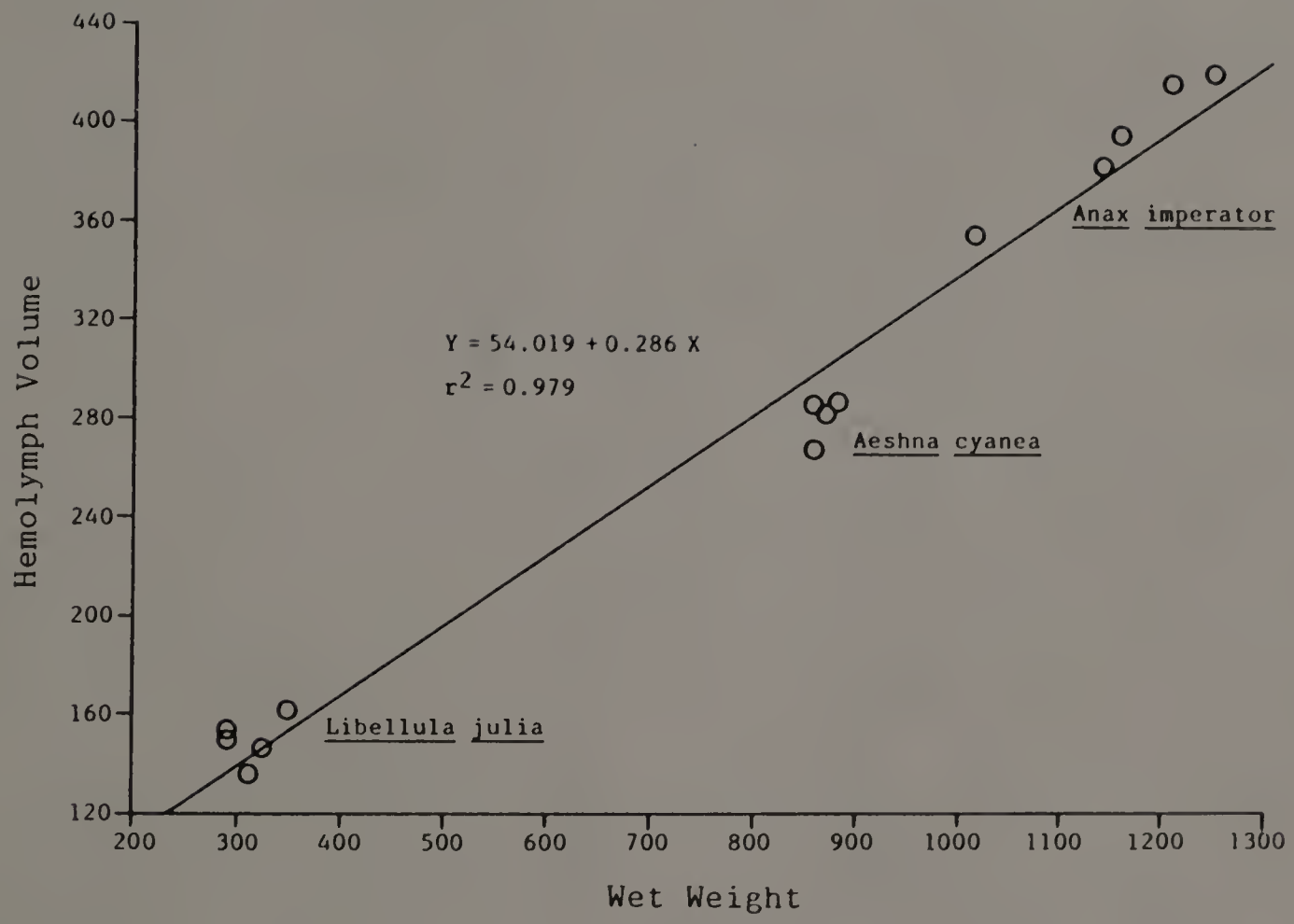

Figure 6. Relationship between wet weight (mg) and hemolymph volume (ul) in last instar nymphs of three dragonfly species. Values for L. julia from the control group; values for A. cyanea and A. imperator from Moens (1975a).

A 96 hour exposure to aluminum (30 mg/1) at very low pH (2.3), in comparison to very low pH alone (2.3/0), resulted in significant reductions of wet and ash weights, body burdens of Na^+ and Ca^{2+}, hemolymph volume and osmolality, and hemolymph Na^+ and Cl^- concentrations. With the exceptions of wet and ash weights and hemolymph osmolality, the 2.3/0 treatment levels were in no instance significantly different from those observed in the control. None of the pH 4.0 treatments provoked responses significantly different from those seen in the control for any of the tested parameters.

Alterations in water balance, as reflected by changes in hemolymph volume and wet weight (Tables 2 and 11), were observed in L. julia as a consequence of low pH/aluminum exposure. Hemolymph volume and wet weight losses of 12 ul/7.6 mg and 63 ul/30.4 mg relative to the control were observed for nymphs in the 2.3/0 and 2.3/30 treatments, respectively. The measured wet weight losses were not sufficient, however, to account for the observed hemolymph volume reductions. It is reasonable to conclude, therefore, that the depressed hemolymph volumes resulted from a loss of water to the environment as well as from a shift of fluid from the hemolymph to the intracellular compartment. A similar redistribution of body water, from the extracellular (plasma and interstitial) compartment to the intracellular compartment, was reported for fish exposed to acidic water, although the total body water content was maintained (McDonald and Wood, 1981; Milligan and Wood, 1982; Lee et al., 1983; Hobe, 1987).

The fluid shift can be viewed as a consequence of plasma ion loss across the gills. This generates osmotic and ionic gradients favoring the movement of water into the intracellular compartment and the movement of electrolytes in the opposite direction (McDonald and Wood, 1981; Milligan and Wood, 1982).

Similarly, Carter (1980) found that larvae of the blackfly Simulium ornatipes exposed to acutely toxic concentrations of zinc (150 mg/l) experienced a reduction in wet weight resulting from a greatly depressed hemolymph volume. He proposed that mortality resulted from dehydration due to increased urine production by the Malpighian tubules with an insufficient compensatory increase in water resorption by the hindgut. Perhaps a similar mechanism was responsible for the altered water balance observed in L. julia.

Disrupted ionic regulation was also observed in nymphs of L. julia exposed for 96 hours to very low pH/aluminum treatments. Specifically, the 2.3/30 treatment effected significantly depressed hemolymph concentrations of Na^+ and Cl^- (32% and 28%, respectively) as well as a reduced Na^+ body burden (61%) relative to the control (Tables 4, 7, and 8). Based on the hemolymph volume and Na^+ concentration reductions, roughly 320 ug Na^+/insect was lost from the hemolymph during a 96 hour exposure to the 2.3/30 treatment. This is very similar to the measured body burden loss of 300 ug Na^+/insect. It appears, therefore, that the extracellular compartment (i.e., the hemolymph) was the primary source of the whole body Na^+ loss observed in L. julia as a

consequence of low pH/aluminum exposure. Although hemolymph levels of Na^+ and Cl^- were respectively reduced by 7% and 9% during a 96 hour exposure to the 2.3/0 treatment, these differences were not found to be significant.

In this study, both the 2.3/0 and 2.3/30 treatments provoked significant reductions in hemolymph osmolality and ash weight (Tables 3 and 6). The difference in hemolymph osmolality between the control and 2.3/30 treatment (83 mmol/kg) was largely accounted for by the depressed hemolymph concentrations of Na^+ and Cl^- (85 mmol/l). Only about one-half of the reduction effected by the 2.3/0 treatment (43 mmol/kg), however, was contributed by these ions (23 mmol/l). Similar trends were observed for ash weight. Assuming Cl^- was the major anion associated with Na^+ loss, these ions would largely account for the ash weight difference between the control and the 2.3/30 treatment (800 ug/insect). None of the measured cation body burdens accounted for the difference in ash weight between the control and the 2.3/0 treatment.

In general, these results compare favorably with those of Berrill et al. (1987) who reported that the levels of Na^+ and Cl^- in the dragonfly Libellula lydia and the damselfly Ischnura verticalis appeared to be unaffected by a 96 hour exposure to water at pH 3.5. Similarly, hemolymph concentrations of Na^+ and Cl^- in the waterbug Corixa punctata were not significantly altered by a 24 to 72 hour exposure to pH 3.0 (Vangenechten and Vanderborght, 1980). In contrast, Na^+ losses were observed in the stonefly Pteronarcys proteus exposed for

72 hours to pH 3.0 (Lechleitner et al., 1985), while both Na^+ and Cl^- losses were reported for the mayflies Stenonema femoratum (Rowe et al., 1988a) and Leptophlebia cupida (Berrill et al., 1987) exposed for 96 hours to pH 3.5. Furthermore, exposure for 72 hours to pH 3.7 resulted in significantly depressed hemolymph concentrations of both Na^+ and Cl^- in the crayfish Astacus astacus (Appelberg, 1985).

Potts and Fryer (1979) noted that the acid tolerant crustacean Acantholeberis curvirostris was able to maintain its Na^+ at pH 3.0, while the acid sensitive Daphnia magna was not. Likewise, both juvenile and adult Cambarus robustus, an acid tolerant crayfish, were able to regulate their internal Na^+ levels at pH 3.8 for 96 hours, whereas juvenile and adult Orconectes rusticus, an acid sensitive crayfish, were not (Hollett et al., 1986; Wood and Rogano, 1986). Furthermore, Havas and Hutchinson (1983) reported net losses of Na^+ and Cl^- from four acid sensitive crustaceans (Daphnia middendorffiana, Diaptomus arcticus, Branchinecta paludosa, and Lepidurus arcticus), as well as from the midge Orthocladius consobrinus, while the more tolerant caddisfly Limnephilus pallens and midge Chironomus riparius showed no such loss during a 48 hour exposure to water at pH 3.0.

Havas and Hutchinson (1983) found lower levels of Na^+ and Cl^- in individuals of Daphnia middendorffiana, Diaptomus arcticus, and Branchinecta paludosa exposed for 48 hours to aluminum (18 mg/l) at low pH, than in those exposed in reference

water at the same pH. These researchers also noted that disrupted Na^+ balance as well as mortality of Limnephilus pallens occurred only following exposure to aluminum at pH 2.8. Similarly, a 14 day exposure to aluminum levels of 250 to 1000 ug/l at pH 5.0 resulted in significantly reduced Na^+ concentrations relative to the day 0 value in the crayfish Pacifastacus leniusculus and Astacus astacus (Appelberg, 1985). Exposure of the mayflies Ephemera danica and Heptagenia sulphurea to 0.5 and 2.0 mg inorganic monomeric Al/l at pH 4.0 and 4.8 for 14 days resulted in depressed Na^+ body burdens, compared to low pH alone (Herrmann, 1987b). In contrast, slightly elevated hemolymph Na^+ concentrations in the waterbug Corixa punctata were observed as a consequence of a 20 hour exposure to 10 and 50 mg Al/l at pH 4.0 (Witters et al., 1984). It is interesting to note that slightly elevated hemolymph ion concentrations were also observed in this study for L. julia following a 96 hour exposure to the pH 4.0 (0, 0.3, and 30 mg Al/l) treatments.

In L. julia, the body burdens of K^+ and Mg^{2+} and the hemolymph concentrations of K^+, Ca^{2+}, and Mg^{2+} were not found to be significantly affected by a 96 hour exposure to any of the treatments (Tables 4, 5, 8, and 9). The Ca^{2+} body burden, however, was found to be significantly lower in the 2.3/30 treatment than in the 2.3/0 treatment, but not significantly reduced relative to the control. These results are in agreement with Lechleitner et al. (1985) who found no significant changes in the body burdens of K^+, Ca^{2+}, and Mg^{2+} in the stonefly

Pteronarcys proteus following a 5 day exposure to pH 3.0. Similarly, a 96 hour exposure to pH 3.5 resulted in no significant change in the whole body K^+ concentration of the mayfly Stenonema femoratum (Rowe et al., 1988a). In contrast, the caddisfly Limnephilus pallens exposed for 48 hours to pH 3.0, as well as the crustaceans Daphnia middendorffiana, Diaptomus arcticus, Branchinecta paludosa, and Lepidurus arcticus exposed to pHs of 4.5 or less for 48 hours, experienced net losses of Ca^{2+} (Havas and Hutchinson, 1983). In addition, these researchers noted greater Ca^{2+} losses by individuals of these species exposed for 48 hours to 18 mg Al/l at low pH, than in those exposed in reference water at the same pH.

Despite the substantial hemolymph volume reduction observed in the 2.3/30 treatment (63 ul), the concentrations of K^+, Ca^{2+}, and Mg^{2+} in the hemolymph, although slightly elevated, were not significantly altered. The hemolymph concentrations of these cations were regulated and since the body burdens, with the exception of Ca^{2+}, were maintained (i.e., not lost to the environment), it would appear that a shift of these ions similar to that previously described for water, from the hemolymph to the intracellular compartment, occurred. It is difficult to evaluate these changes, however, because it is not clear to what extent they are adaptive.

In contrast with the 96 hour results, a 192 hour exposure to pH 2.3 with 0, 0.3, and 3 mg Al/l elicited significant alterations in the hemolymph concentrations of both Na^+ and K^+ (Table 10).

The hemolymph Na^+ levels in these treatments were all reduced by roughly 60%, while the K^+ concentrations were 2.4 to 3.1 fold higher than those found in the control. The regression of hemolymph K^+ on Na^+ for the three treatments is shown in Figure 7. Based on this, it appears that the nymphs were able to successfully regulate their hemolymph K^+ levels at 4 mmol/l until their hemolymph Na^+ concentrations were reduced to roughly 75 mmol/l (i.e., the value for Na^+ where K^+ equals 4 mmol/l). Supporting this, the mean hemolymph Na^+ and K^+ concentrations in nymphs exposed for 96 hours to the 2.3/30 treatment were 104 and 4.1 mmol/l, respectively. It must be pointed out, however, that the relationship observed between these ions could be coincidental because in the dragonfly Libellula quadrimaculata, hemolymph Na^+ and K^+ levels were found to be regulated by independent mechanisms (Nicholls, 1983).

A 192 hour exposure to aluminum at concentrations of 0.3 and 3 mg/l resulted in no additional toxicity, as indicated by hemolymph Na^+ and K^+ concentrations, beyond that found for the 2.3/0 treatment. In the 96 hour experiments as well, low levels of aluminum (0.3 and 3 mg/l) at pH 2.3 were found to provoke no significant additional effect, compared to those of the 2.3/0 treatment, on wet weight and hemolymph cation concentrations. These results are consistent with those of the subchronic toxicity test (Table 1, Figure 2), where no significant differences were found among the median survival times for treatments with 0, 0.5, and 5 mg Al/l at pH 2.4.

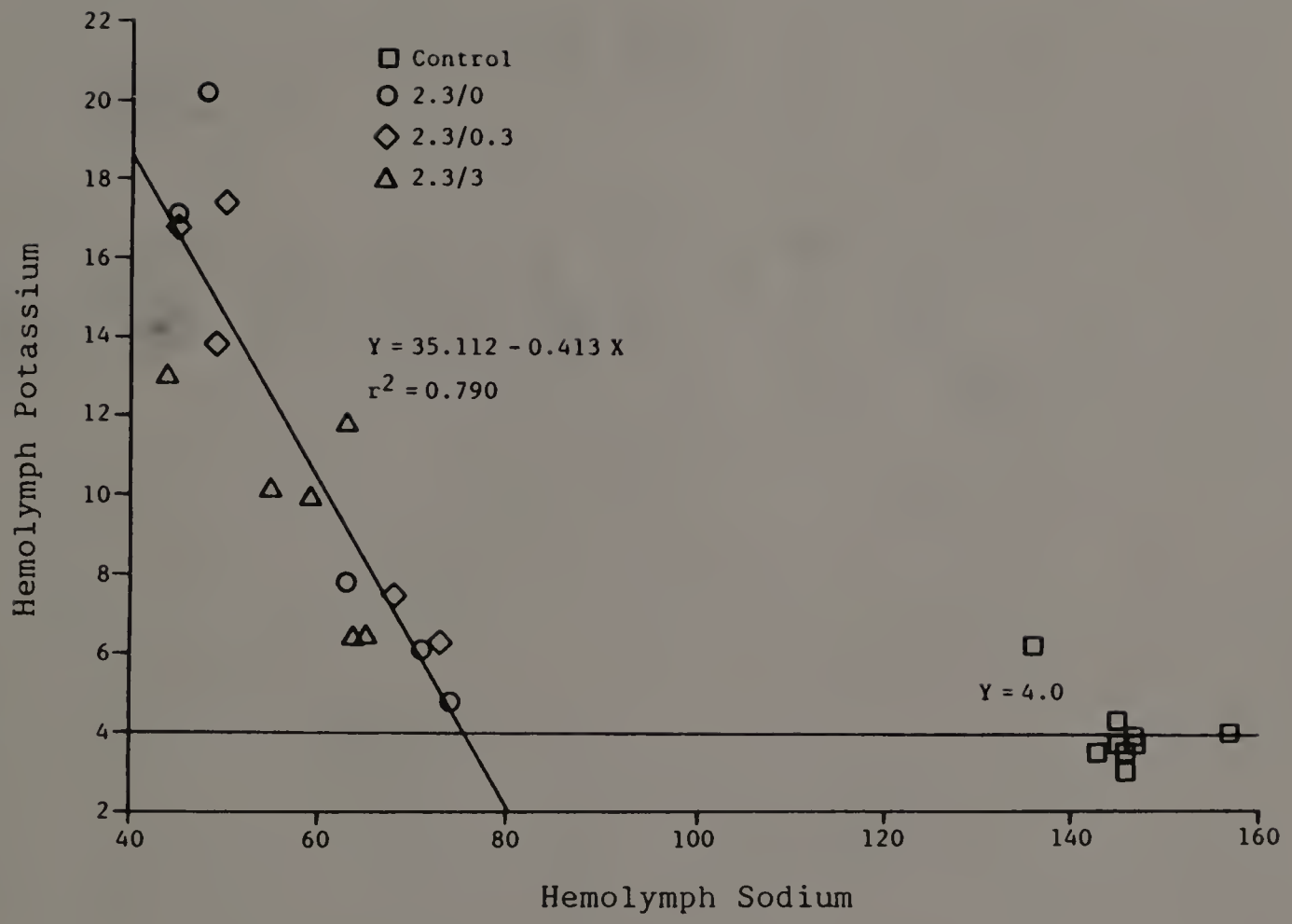

Figure 7. Relationship between hemolymph concentrations of sodium and potassium (mmol/l) following a 192 hour exposure to aluminum at pH 2.3. The legend shows nominal pH/Al (mg/l) levels. See text for further explanation.

Acid-Base Balance -- Hemolymph pH

The pH of insect hemolymph generally lies between 6.0 and 7.5 (Sutcliffe, 1962), and in most species has a slightly acid (6.4 to 6.8) reaction (Wyatt, 1961). The hemolymph of dragonfly nymphs, however, is more alkaline with values of 7.6 and 8.2 reported for Somatochlora cingulata and Uropetala carovei, respectively (Correa et al., 1985; Bedford and Leader, 1975). The mean control value obtained in this study for L. julia (7.58) was nearly identical to that observed for S. cingulata.

In L. julia, hemolymph pH levels were found to be affected by a 96 hour exposure to very low pH and elevated aluminum concentrations at low pH (Table 12). A 0.2 unit reduction relative to the control was elicited by the 2.3/0, 2.3/0.3, 2.3/3, and 4.0/30 treatments, while a 0.8 unit depression was provoked by the 2.3/30 treatment. Similarly, a 0.6 to 0.8 unit reduction in hemolymph pH was observed in the midges Chironomus riparius (from Uppsala) and Chironomus plumosus following a 7 day exposure to pH 3.5 (Jernelov et al., 1981). Likewise, a severe metabolic acidosis (0.5 units) developed in the crayfish Orconectes propinquus following a 5 day exposure in soft water at pH 4.0 (Wood and Rogano, 1986). In the latter case, the pH depression was attributed to a large uptake of acidic equivalents from the environment. In contrast, a 0.5 to 0.6 unit rise in hemolymph pH, as a consequence of elevated hemolymph ammonia levels, was reported for nymphs of the dragonfly Somatochlora cingulata exposed for 96 hours to pH 3.59 and to 30 mg Al/l at pH

4.2 (Correa et al., 1985). The observed disparity in the responses of L. julia and S. cingulata to very similar stimuli (4.0/30 and 4.2/30) remains unexplained.

Oxygen Consumption

In L. julia, respiration rates decreased with increasing concentrations of aluminum at pH 4.0 (Figure 4). While a reduction in the mean oxygen consumption was observed in all of the treatments, this difference was significant relative to the control only when the aluminum concentration equaled or exceeded 3 mg/l. These observations are in general agreement with those of Correa et al. (1985) for the dragonfly Somatochlora cingulata. In both of these species, elevated concentrations of hydrogen ions and aluminum were found to depress oxygen consumption; the primary difference between them being a matter of degree and the relative importance of low pH versus aluminum in eliciting the response. In S. cingulata, elevated aluminum concentrations (10 to 30 mg/l at pH 4.2) did not provoke a significant change in respiration compared to low pH alone, while in L. julia, the opposite was true (i.e., oxygen uptake in the 4.0/30 treatment was significantly lower than in the 4.0/0 treatment). Larvae of the caddisfly Limnephilus sp. exposed for 96 hours to environmentally relevant pH/aluminum levels (4.0/0 and 4.0/0.3) showed no significant change in oxygen consumption (Correa et al., 1986). Oxygen consumption by L. julia exposed to the same conditions,

although reduced by 28% and 36%, respectively, was also not significantly depressed relative to the control.

In contrast, Herrmann and Andersson (1986) reported significantly increased oxygen uptake rates relative to those observed for low pH alone in the mayflies Ephemera danica, Heptagenia fuscogrisea, and Heptagenia sulphurea following a 10 day exposure to 0.5 and 2.0 mg inorganic Al/l at pH 4.0 and 4.8. The proffered explanation was that mucus and/or aluminum hydroxide precipitates physically interfered with oxygen uptake (mechanical impact route), and/or impaired osmoregulation together with other toxic effects reduced respiratory efficiency (chemical impact route), resulting in an increased compensatory respiration rate (Herrmann and Andersson, 1986; Herrmann, 1987a). It seems incongruous, however, to conclude that a stressed organism would be able to overcompensate and take up more oxygen through the very system being impacted.

Assuming that the metabolic costs of homeostasis are reflected in oxygen consumption, an increased respiration rate would be expected in response to an osmoregulatory stress. It has been shown, however, that this assumption does not apply to nymphs of the stonefly Paragnetina media and larvae of the mosquito Aedes aegypti exposed to extreme salinities (Kapoor, 1979; Edwards, 1982). In both of these species, the metabolic energy demand for osmoregulation was considered minimal as no significant differences were found among the oxygen uptake rates.

Although reduced oxygen carrying capacity of the blood has been observed in fish (Packer, 1979), it seems unlikely that this would be affected in insects because air filled trachea and tracheoles, rather than a blood pigment (i.e., hemoglobin), deliver oxygen to the cells. Because the hemolymph of insects is generally not concerned with oxygen transport (Florkin and Jeuniaux, 1964), hemolymph acidosis should have little or no effect on the distribution of oxygen to the tissues.

Reduced oxygen uptake in aquatic organisms has also been attributed to mechanical blockage of the respiratory surfaces by one or more of the following: accumulated/denatured mucus, precipitated aluminum hydroxide, and epithelial cell damage. In fish, mucus, formed during exposure to acid, reduced oxygen uptake by increasing the water to blood diffusion distance and by forming a nonconvective layer which inhibited water flow between the gill lamellae (Ultsch and Gros, 1979). This response is similar to that described by Carpenter (1927) for heavy metals and is known as the coagulation film anoxia theory (Westfall, 1945).

No evidence of mucus production or accumulation was observed in the stonefly Pteronarcys dorsata exposed to low pH (Lechleitner et al., 1985) or in the mayflies Ecdyonurus venosus and Baetis rhodani exposed to aluminum at low pH (McCahon et al., 1987). In this regard, Lechleitner et al. (1985) speculated that the cuticle of the gill tissue acts as a protective layer that doesn´t require mucus.

Aluminum has been found to be associated with the respiratory and osmoregulatory surfaces of both fish and macroinvertebrates. Trace quantities of aluminum were found on the surfaces of the chloride cells and respiratory epithelia as well as within membrane bound, cytoplasmic vacuoles within the gill epithelium of fish exposed to this metal (Neville, 1985; Karlsson-Norrgren et al., 1986; Youson and Neville, 1987). The latter researchers speculated that the accumulation of aluminum on the cell surfaces interfered with respiratory gas exchange.

In the mayflies Ecdyonurus venosus and Baetis rhodani exposed to 350 ug Al/l at pH 5 for 24 hours, aluminum was observed over the entire exoskeleton, with the staining reaction for E. venosus greatest in the gut, within and surrounding the gill plates, and on the outer surface of the abdomen (McCahon et al., 1987). Furthermore, opaque material (thought to be either mucus or aluminum precipitate) was observed on the gills and other body surfaces of the mayflies Heptagenia fuscogrisea, Heptagenia sulphurea, and Ephemera danica following exposure to 0.5 and 2.0 mg Al/l at pH 4.0 and 4.8 for 10 days (Herrmann and Andersson, 1986). Citing the results of the latter researchers, McCahon et al. (1987) postulated that aluminum physically occludes the principal respiratory surface, the integument, resulting in an increased respiration rate.

Fish exposed to low pH or aluminum at low pH showed extensive gill tissue damage (edema, necrosis, hyperplasia, lamellar fusion, separation of epithelium from the underlying tissue, and

degeneration of the chloride cells) (Daye and Garside, 1976; Chevalier et al., 1985) resulting in an increased diffusion distance and a reduced water space (Karlsson-Norrgren et al., 1986). Like mucus and aluminum accumulation, damage to the gill lamellae is expected to interfere with oxygen uptake (McDonald, 1983; Tietge et al., 1988).

In this regard, oxygen consumption by the mayfly Isonychia bicolor exposed to an alkaline solution (pH 11) was believed to be hindered by the accumulation of fluid in the osmoregulatory cells of the gill tissue (Peters et al., 1985). A similar histological response (i.e., vacuole formation) was observed in the chloride cells of the gills of the stonefly Pteronarcys dorsata exposed for 9 hours to pH 2.5 (Lechleitner et al., 1985). These researchers also noted that a 96 hour exposure to pH 4.0 resulted in subtle changes in the gill tissue and chloride cells. Although histological evidence is lacking, it is possible that damage to the gill tissue, together with aluminum and/or mucus (if produced) accumulation, could account for the depressed oxygen uptake observed in L. julia.

CHAPTER VI

CONCLUSIONS AND RECOMMENDATIONS

Conclusions

Several conclusions may be drawn regarding the tolerance and physiological responses of last instar nymphs of *Libellula julia* exposed to low pH and aluminum at low pH.

1) The nymphs were extremely tolerant of both low pH and elevated aluminum concentrations at low pH.
2) The toxic effects of hydrogen ions and aluminum are very similar. To some extent, both were found to impact acid-base status, oxygen consumption, water balance, and ionic regulation.
3) Significantly depressed hemolymph pH levels were found in nymphs exposed for 96 hours to very low pH (2.3) alone or with added aluminum (0.3, 3, and 30 mg/l) and to low pH (4.0) with a greatly elevated aluminum level (30 mg/l).
4) A significant decrease in oxygen uptake was observed in nymphs exposed to sublethal aluminum concentrations (3 and 30 mg/l) at pH 4.0. Of the various methodologies used to evaluate stress, this proved to be the most sensitive.
5) The extreme tolerance to low pH and aluminum demonstrated by *L. julia* was reflected in its water and ion regulation physiology. Disrupted ionic regulation and alterations in water balance were observed following 72 to 192 hour

exposures to very low pH (2.3) and aluminum (0.3, 3, and 30 mg/l) at very low pH. At pH 4.0, however, no significant changes in any of the tested parameters were observed, even when the aluminum concentration was 30 mg/l.

6) Since at pH 2.3 (and to a lesser extent at pH 4.0) aluminum exists primarily as the ion and as acid stable complexes, it would seem that a biological impact can be exerted not only by the ionized and nonionized hydroxides, but also by Al^{3+}.

7) Based upon the close correlation between hemolymph Na^{+} (Cl^{-}) concentrations and the subchronic toxicity test results, it appears that the primary cause of mortality in L. julia, as a consequence of very low pH/aluminum exposure, was the disruption of ionic regulation.

Recommendations

The survey of the physiological responses of L. julia to low pH and aluminum at low pH has touched upon a number of areas worthy of continued research. These include:

1) The underlying mechanisms responsible for the net Na^{+} and Cl^{-} losses observed in nymphs under low pH/aluminum stress should be identified and the relative importance of each (active influx versus passive efflux) quantified.

2) A histological examination of the excretory/respiratory system (i.e., Malpighian tubules, ileum, and branchial

chamber) should be implemented to determine if these toxicants provoke changes in tissue ultrastructure.

3) The accumulation of aluminum and mucus (if produced) on the general body surface and within the alimentary canal (particularly the branchial chamber) should be investigated using aluminum and mucus specific stains.

4) A more detailed analysis of acid-base status should be implemented. Determination of total CO_2, HCO_3^-, and lactic acid concentrations in the hemolymph may help to identify the origin (i.e., respiratory versus metabolic) of the hemolymph acidosis observed in L. julia.

5) The physiological responses following chronic (30 day) exposures to environmentally relevant pH/aluminum levels should be examined.

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